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Docket No.: 02427/100G772-US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Erich HOFFMANN

Serial No.:	09/844,517	Group Art Unit:	1648
Confirmation No.:	9063	Examiner:	Myron G. Hill
Filed:	April 27, 2001		

For: DNA TRANSFECTION SYSTEM FOR THE GENERATION OF INFECTIOUS INFLUENZA VIRUS

DECLARATION OF ROBERT G. WEBSTER UNDER 37 CFR § 1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

I, ROBERT G. WEBSTER, do hereby declare and state the following:

1. I am a Director of the World Health Organization (WHO) Collaborating Center for Studies on the Ecology of Influenza in Animals and Birds, Member of the Division of Virology, Department of Infectious Diseases and Rose Marie Thomas Chair at St. Jude Children's Research Hospital, Professor at the Department of Microbiology and Immunology at the University of Tennessee Center for the Health Sciences, Memphis, and Visiting Distinguished Professor of Excellence at the Department of Microbiology at the University of Hong Kong, Hong Kong SAR.

2. I was awarded a Ph.D. degree in Microbiology in 1962 from the Australian National University, Canberra. Upon completion of my doctorate, I worked as a Research Fellow (Fullbright Scholar) at the Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor and then as a Fellow at the Department of Microbiology, John Curtin Medical School, Australian National University, Canberra. In 1968, I joined the Laboratories of Virology and Immunology at St. Jude Children's Research Hospital and Department of Microbiology at University of Tennessee, Memphis as an Assistant Professor. After obtaining a Member position in 1969, I have been continuously affiliated with St. Jude Children's Research Hospital, where I currently hold the title of Member and Rose Marie Thomas Chair at the Division of Virology, Department of Infectious Diseases.

3. During my entire career I have been working in the field of virology, with a specific focus on influenza virus biology and production of recombinant vaccines. My contributions to the field of influenza virology have been acknowledged by numerous international awards and honorary titles, which include, among others, a title of the Director of the WHO Collaborating Center, as noted above, Member of the National Academy of Sciences

of the United States, Fellow of the Royal Society of London, Fellow of the Royal Society of New Zealand, and holder of the 12th Annual Bristol-Myers Squibb Award for Distinguished Achievement in Infectious Diseases 2002. A copy of my Curriculum Vitae is annexed as Exhibit 1.

4. My contributions to the field of influenza virology are disclosed in numerous patents and scientific and popular publications, which include, among others, U.S. Patents No. 6,344,354; 5,916,879; 5,824,536; 5,643,578; 4,552,758; 4,552,757 and published U.S. Application No. 2004/0142450. I have also authored a number of widely used textbooks on virology and, in particular, influenza virology, which include, among others, *Textbook of Influenza*, Karl G. Nicholson, Robert G. Webster, Alan J. Hay, Blackwell Publishers, 1998; *Encyclopedia of Virology*, Robert G. Webster, Allan Granoff, Academic Press, 1999, 1996 and 1994; *Origin and Evolution of Viruses*, Esteban Domingo, Robert G. Webster, John J. Holland, Academic Press, 1999.

5. My contributions to the area of influenza vaccine production include, among others, development of a mild detergent treatment of whole virus vaccines. This treatment reduces vaccine toxicity and this type of approach is currently used to produce all influenza vaccines used throughout the world.

6. Many leading world experts in the field of influenza virology have spent time in my laboratory at St. Jude Children's Research Hospital training as graduate students or postdoctoral fellows or obtaining various types of expertise as visiting scientists. The list of such

experts includes, among others, Dr. Yoshihiro Kawaoka[1] (who was a postdoctoral fellow in my laboratory 08/83-06/85 and then an Assistant and Associate Member 07/85-06/97), Dr. Gabriele Neumann[2] (who was a postdoctoral fellow in my laboratory 08/95-06/97), and Dr. Erich Hoffmann[3] (who was a postdoctoral fellow in my laboratory 03/98-08/01).

7. I make this Declaration in support of the application identified above ("the '517 application"). I am not an inventor of the '517 application and I do not have any financial interest in this application. However, I have a firsthand knowledge of the work that lead to the present invention as this work was conducted in my laboratory at St. Jude Children's Research Hospital.

8. I have reviewed the entire disclosure of the '517 application, including the original claims and the drawings, as well as the prosecution history and claims, as amended in the accompanying amendment. I have also specifically reviewed the Final Office Action dated May 5, 2004, which was issued in connection with this application.

9. I understand that in the Final Office Action dated May 5, 2004, the United States Patent and Trademark Office has rejected claims 15-17, 19-30, 32, 39, and 44 of the '517 application under 35 U.S.C. § 103(a) on the grounds that the claims are allegedly obvious over Hoffmann dissertation (1997) and Neumann *et al.* (Proc. Natl. Acad. Sci., 1999, 96: 9345-50) and has rejected claim 45 as being allegedly obvious over Hoffmann and Neumann *et al.* and further in view of Pleshka *et al.* (J. Virol., 1996, 70: 4188-92).

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[1] The senior author of the Neumann *et al.* article cited by the Examiner.
[2] The lead author of the Neumann *et al.* article cited by the Examiner.
[3] The sole inventor of the above-identified patent application (the '517 application).

10. In the Office Action, the Examiner has stated that, knowing that Neumann *et al.* (i) generated infectious influenza virus using a plasmid-based system, and (ii) disclosed the benefit of adding more protein expressing plasmids to the transfection, and also knowing that multi-plasmid transfections are complex, one of ordinary skill in the art at the time of the invention would be motivated to come up with the present invention by using the plasmid described in the Hoffmann dissertation to reduce the number of plasmids for transfection and to save time in cloning. The Examiner seems to believe that there was an expectation of success, because the promoter elements used by Hoffmann are the same as used by Neumann *et al.*

11. Based on the materials I reviewed, my extensive experience in the field, and my firsthand knowledge of the work that lead to Neumann *et al.* publication and to the present invention[4], it is my opinion that the Hoffmann dissertation and Neumann *et al.* article do not suggest or provide any leads for the creation of the dual pol I-pol II promoter plasmid system for the generation of infectious influenza viruses from cloned viral segments as disclosed in the '517 application and recited in the present claims. Much less do these cited references provide any expectation that the generation of an infectious influenza virus could be successfully accomplished using the 8-plasmid pol I-pol II system of the present invention. The detailed explanation is provided below.

12. First, I would like to describe the historical context in which the present invention came into existence. The antigenic variation of influenza A virus hemagglutinin (HA) and

[4] As specified in Section 6, above, both Dr. Gabriele Neumann (the lead author of the Neumann *et al.* article cited by the Examiner) and Dr. Erich Hoffmann (the sole inventor of the '517 application) were postdoctoral fellows in my laboratory. Dr. Neumann was a postdoctoral fellow in my laboratory from August 1995 to June 1997, and Dr. Hoffmann was a postdoctoral fellow in my laboratory from March 1998 to August 2001.

neuraminidase (NA) surface glycoproteins requires very frequent changes in vaccine formulations. The classical method of creating influenza virus seed strains for vaccine production generates 6 + 2 reassortants that contain six genes from a high-yield laboratory-adapted avirulent virus, such as A/PR/8/34 (H1N1), and the HA and NA genes of the circulating strains. The resulting reassortant virus has the antigenic properties of the circulating strain and the safety and high-yield properties of the laboratory-adapted virus. Reassortants are currently generated by coinfecting the high-yield laboratory-adapted virus with the circulating virus. Coinfection with two influenza viruses containing 8 segments can theoretically result in the generation of 2^8-2=254 different progeny viruses. Clearly, this method is extremely time-consuming because of the selection process required to isolate the desired reassortant virus. Reverse genetics could significantly speed this process by generating a set of plasmids encoding the internal genes of a high-yield virus. Recent re-emergence of human infections caused by avian influenza viruses (*e.g.*, the avian H5N1 influenza virus, A/Hong Kong/213/03) which (i) have no antigenically similar avirulent viruses and (ii) kill embryonated chicken eggs (where the seed strains are normally propagated), have further increased the need for rapid and reproducible plasmid-based reverse genetic approaches to influenza vaccine development and production.

13. Neumann *et al.* (1999 publication cited by the Examiner) described a first reverse genetic system for influenza virus production (a very similar system was also developed by Fodor *et al.*, J. Virol., 1999, 73: 9679-82, attached as Exhibit 2). The system of Neumann *et al.* contains two types of plasmids: (i) pol I-only replication plasmids directing the synthesis of vRNAs from a pol I promoter and (ii) pol II-only protein expression plasmids encoding viral proteins (*i.e.*, at least plasmids encoding viral polymerase proteins PB1, PB2, PA, and NP). Accordingly, this reverse genetic system uses the total number of plasmids which exceeds the

total number of gene segments from the source virus (*i.e.*, at least 12 plasmids for an 8-segmented influenza A virus). The separation of pol I and pol II promoters on different plasmids lies at the very core of the Neumann *et al.* system, as this system tries to recapitulate the life cycle of an influenza virus by allowing regulation of viral protein expression separately from viral genome segment replication. In fact, in its most efficient (high yield) versions, the system of Neumann *et al.* uses extra protein expression (pol II) plasmids encoding HA, NA, M1, M2, and NS2 viral proteins (making the total number of transfected plasmids 17 instead of 12; see, p. 9347 [¶ bridging left and right col.] and Table 1 at p. 9348 of the Neumann *et al.* article). The optimal use of Neumann *et al.* system also relies on the ability to provide specified amounts of each viral protein by transfecting different amounts of each of the protein expression (pol II) plasmids (see, *e.g.*, p. 9347 left col., ¶¶ 3-4 of the Neumann *et al.* article).

14. The 8-plasmid dual pol I-pol II promoter system of the present invention is an alternative plasmid-based system for generation of an infectious influenza virus entirely from cloned DNA. This system represents not a minor variant of the Neumann *et al.* system as suggested by the Examiner, but a different approach altogether, based on a totally different principle of coordination of viral replication and protein expression. As specified above, in the Neumann *et al.* system, viral segment replication and protein expression are regulated separately due to the use of pol I-only plasmids directing the synthesis of vRNAs from a pol I promoter and pol II-only plasmids directing the synthesis of mRNAs encoding viral proteins from a pol II promoter. In contrast, in the dual pol I-pol II promoter system of the present invention, the vRNA (or cRNA) and mRNA synthesis occur from the same plasmid. As specified above, the ability to separately regulate the amount of viral proteins produced was believed to lie at the very core of the success of the Neumann *et al.* system. Accordingly, when Dr. Hoffmann came up

with the idea of the dual pol I-pol II promoter system, everyone in the laboratory, including myself, was very skeptical that such a system could produce an infectious virus.

15. At the time, we were fully aware of Dr. Hoffmann's prior Ph.D. thesis work on dual pol I-pol II promoter plasmids (the 1997 Hoffmann dissertation cited by the Examiner). However, this work gave no indication whatsoever that an infectious influenza virus could be produced in the absence of any helper virus using eight pol I-pol II plasmids, each encoding one viral segment. The Hoffmann dissertation merely describes single pol I-pol II plasmids carrying reporter genes such as CAT and GFP, which are capable of directing transcription of a single reporter mRNA and expression of a reporter protein using host cell transcription/translation machinery, and replication of a single reporter RNA in the presence of viral polymerase proteins supplied by a helper virus (*e.g.*, FPV). The Hoffmann dissertation does not even provide a suggestion of making a single pol I-pol II plasmid encoding a single viral gene segment, much less a full set of such plasmids to achieve generation of an infectious viral particle in the absence of any helper virus. Accordingly, Dr. Hoffmann's dissertation provided no support for his novel idea of the 8-plasmid dual pol I-pol II promoter system.

16. Although I was skeptical that the 8-plasmid dual pol I-pol II promoter system suggested by Dr. Hoffmann would ever generate an infectious virus, I admired his enthusiasm and believed in his skills as an excellent molecular biologist. I therefore supported his work. To everyone's great surprise and excitement, he succeeded in generating an infectious virus (as disclosed in the '517 application). In fact, the dual pol I-pol II promoter system of the present invention has rapidly become the reverse genetic system of choice in the field of influenza vaccine production. This system turned out to be not only much simpler and more reproducible

than the system of Neumann *et al.*, because it significantly decreased the number of plasmids for transfection and eliminated the need to adjust the relative amount of each plasmid, but efficient as well. Also, in contrast to the Neumann *et al.* system, which for a long time was capable of efficiently producing infectious virus only in 293T cells, the dual pol I-pol II promoter system of the present invention efficiently generated infectious viral particles in monkey Vero cells. This is not a trivial difference. 293T cells are transformed cells that are not approved by WHO for vaccine seed production. Vero cells are approved for vaccine seed production. Thus, at the time Dr. Hoffmann did the work leading to the present invention, the Neumann *et al.* system would have been regarded as not yet practical. Nothing in Hoffmann's thesis indicates this particular characteristic of the dual pol I-pol II promoter system.

17. More and more investigators around the world are using the dual pol I-pol II promoter reverse genetic system of the present invention and prefer it to the system of Neumann *et al.* (see, *e.g.*, a report from the National Influenza Center of Netherlands by de Wit *et al.*, Virus Res., 2004, 103: 155-161; attached as Exhibit 3).

18. We and others have demonstrated the unmatched usefulness of the 8-plasmid dual pol I-pol II promoter system of the present invention for the fast and easy generation of influenza vaccines. For example, in Hoffmann *et al.*, Vaccine, 2002, 20: 3165-3170 (attached as Exhibit 4), Dr. Hoffmann and I, with other researchers at St. Jude Children's Research Hospital, have demonstrated the direct application of the 8-plasmid dual pol I-pol II promoter system to rapid and reproducible generation of reassortant influenza A viruses having the antigenic determinants of the influenza virus strains A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), A/teal/HK/W312 (H6N1), and A/quail/HK/G1/97 (H9N2), and a growth phenotype in

embryonated chicken eggs which was equivalent to that of the wild-type virus. Even more importantly, the use of the 8-plasmid dual pol I-pol II promoter system of the present invention allowed our team at St. Jude to produce a vaccine to the deadly avian H5N1 influenza virus A/Hong Kong/213/03 (which caused human infections and lead to a WHO pandemic alert on February 19, 2003). The vaccine was produced in Good Manufacturing Practice (GMP)-grade facilities *in less than 4 weeks* from the time of virus isolation (see Webby *et al.*, Lancet, 2004, 363: 1099-103; attached as Exhibit 5). The use of the 8-plasmid dual pol I-pol II promoter system allowed us to remove the polybasic amino acids from the HA cleavage site, which are associated with high virulence of the H5N1 virus, and produce a reference vaccine virus on an A/Puerto Rico/8/34 (PR8) backbone in WHO-approved Vero cells. Specifically, using a PCR-based mutagenesis approach, we replaced the cleavage site encoded by the HA gene of H5N1 virus with that of the avirulent A/teal/Hong Kong/W312/97 (H6N1) strain. This modified HA gene and the NA gene of H5N1 were cloned individually into pol I-pol II dual promoter vectors. The two resulting plasmids and the six plasmids encoding the remaining proteins of PR8 were transfected into Vero cells under GMP conditions to rescue the vaccine seed virus. 36–48 hours after transfection, isolated areas of cytopathic effect could be seen on the Vero monolayers. The candidate vaccine strain grew to high titers on subsequent amplification in eggs and did not cause embryo death. The rescued virus was fully sequenced and was identical to the plasmids used in its creation. This virus proved to be non-pathogenic in chickens and ferrets and was shown to be stable after multiple passages in embryonated chicken eggs. The ability to produce a candidate reference virus in such a short period of time has set a new standard for rapid response to emerging infectious disease threats and has clearly demonstrated the superior usefulness of the 8-plasmid dual pol I-pol II promoter reverse genetic system for influenza

vaccine development. The same technologies and procedures are currently being used to create reference vaccine viruses against the 2004 H5N1 viruses circulating in Asia. In sum, the approach set out in the '517 application addresses a raging need for rapid influenza vaccine response, particularly with the expectation of a worldwide pandemic on the order of the 1918 flu pandemic in the near term.

19. In light of the above, it is my opinion that the present invention represents a significant breakthrough both in the field of basic virology of negative stranded RNA viruses and in the field of influenza vaccine production. I strongly disagree with the Examiner's conclusion that the present invention is obvious over the Hoffmann dissertation and Neumann *et al.* article. Perhaps viewed from the vantage of Dr. Hoffmann's success it seems obvious to introduce the viral gene segments into pol I-pol II dual promoter plasmids. However, none of us - including myself, one of the leading experts in influenza in the world, thought so at the time. As noted above, having a full knowledge of the Hoffmann dissertation and Neumann *et al.* article, I along with other colleagues familiar with Dr. Hoffmann's idea were highly skeptical that his invention would ever work and produce an infectious virus. Moreover, a close reading of the Hoffmann's thesis provides no basis to extrapolate his curious reporter gene dual promoter construct even to plasmid-based expression of a single viral mRNA and vRNA. That work led to a separate publication in a leading journal (Hoffmann *et al.*, Virology, 2000, 267: 310-7; attached as Exhibit 6). As noted above, the Neumann *et al.* publication describes an alternative approach, like the present invention based on plasmids to generate an infectious influenza virus, but otherwise having nothing in common with the system of the present invention. It took extraordinary vision to employ the dual pol I-pol II promoter system for all of the viral segments. In my view, such vision, and the results obtained, are the antithesis of obviousness.

20. I hereby declare that all statements made herein of my own knowledge are true and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

10-19-04

Date:

Robert G. Webster, Ph.D.

CURRICULUM VITAE

NAME: ROBERT GORDON WEBSTER

CITIZENSHIP: American

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Division of Virology
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Memphis, Tennessee 38105-2794
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ACADEMIC DEGREES:

BSc	1955	Otago University, New Zealand (Microbiology)
MSc	1957	Otago University, New Zealand (Microbiology)
PhD	1962	Australian National University, Canberra (Microbiology)

PROFESSIONAL APPOINTMENTS:

1958-59	Virologist, New Zealand Department of Agriculture
1962-63	Postdoctoral Fellow, Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor (Fullbright Scholar)
1964-66	Research Fellow, Department of Microbiology, John Curtin Medical School, Australian National University, Canberra
1966-67	Fellow, Department of Microbiology, John Curtin Medical School, Australian National University, Canberra
1968-69	Associate Member, Laboratory of Immunology, St. Jude Children's Research Hospital, and Associate Professor, Department of Microbiology, University of Tennessee Medical Units, Memphis
1969-74	Member, Laboratories of Virology and Immunology, St. Jude Children's Research Hospital, and Associate Professor, Department of Microbiology, University of Tennessee Medical Units, Memphis
1974-75	Member, Laboratories of Virology and Immunology, St. Jude Children's Research Hospital, and Professor, Department of Microbiology, University of Tennessee Center for the Health Sciences, Memphis
1975-78	Member, Division of Virology, St. Jude Children's Research Hospital, and Professor, Department of Microbiology, University of Tennessee Center for the Health Sciences, Memphis
1978-79	Fogarty International Senior Fellow, National Institute for Medical Research, Medical Research Council, London, England
1978-85	Member, Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, and Professor, Department of Microbiology and Immunology, University of Tennessee Center for the Health Sciences, Memphis
1985 to 2001	Member, Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee
1988 to 2001	Rose Marie Thomas Chair, Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, Tennessee
2001 to present	Member, Rose Marie Thomas Chair, Division of Virology, Department of Infectious Diseases, St. Jude Children's Research Hospital, Memphis, Tennessee

APPOINTMENTS:

1975 to Present	World Health Organization Designation as a WHO collaborating laboratory on the ecology of influenza viruses in lower animals and birds

PROFESSIONAL SOCIETY MEMBERSHIPS:

American Society for Microbiology
American Society for Virology
Fellow of the Royal Society of Medicine
American Association for the Advancement of Science

HONORS:

Fellow of the Royal Society, London, 1989
Fellow of the Royal Society of New Zealand, 1990 (Honorary)
National Academy of Sciences of the United States of America, 1998
Twelfth Annual Bristol-Myers Squibb Award for Distinguished Achievement in Infectious Diseases, 2002

RESEARCH INTERESTS:

The emergence and control of influenza viruses
Viral immunology

PUBLICATIONS:

Original Articles

1. Webster RG, Miles JAR. The relations of contagious pustular dermatitis virus. Proc Univ Otago Med Sch 35:2-3, 1957.

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12. Webster RG, Laver WG. Antigenic determinants on the hemagglutinin subunits of influenza A viruses and their role in immunity. In: Laver WG, Bachmayer H, Weil R, eds. The influenza virus hemagglutinin: Topics in infectious diseases. Vol 3. New York: Springer-Verlag, pp 1-14, 1977.

13. Laver WG, Webster RG. 'N-Terminal amino acid analysis of hemagglutinin molecules from duck and equine influenza viruses previously implicated as progenitors of the Hong Kong strain of human influenza. In: Laver WG, Bachmayer B, Weil R, eds. The influenza virus hemagglutinin: Topics in infectious diseases. Vol 3. New York: Springer-Verlag, pp 139-166, 1977.

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15. Webster RG. The evolution of epidemic influenza viruses. In: Bryans JT, Gerber H, eds. Equine infectious diseases IV. Proceedings of the 4th international conference on equine infectious diseases. Princeton, New Jersey: Veterinary Publications, pp 305-313, 1978.

16. Webster RG, Bean WJ Jr. Genetics of influenza virus. In: Roman HL, ed. Annual review of genetics. Vol 12. Palo Alto, California: Annual Reviews, pp 415-431, 1978.

17. Webster RG, Bean WJ Jr, Hinshaw VS. The ecology of influenza viruses. In: Loutit MW, Miles JAR, eds. Microbial ecology. Berlin: Springer-Verlag, pp 188-193, 1978.

18. Laver WG, Air GM, Webster RG, Gerhard W, Ward CW, Dopheide TA. The mechanism of antigenic drift in influenza virus sequence changes in the hemagglutinin of variants selected with monoclonal hybridoma antibodies. Philos Trans R Soc Lond [Biol] 288:313-326, 1980.

19. Webster RG, Hinshaw VS, Bean WJ, Sriram G. Influenza viruses. Transmission between species. Philos Trans R Soc Lond [Biol] 288:439-447, 1980.

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Collaborative Center for the Collection and Evaluation of Data on Comprehensive Virology, Munich, Germany, 1981.

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26. Laver WG, Air GM, Webster RG. Towards a universal influenza vaccine. In: Bishop DHL, Compans RW, eds. The replication of negative strand viruses. New York: Elsevier/North-Holland, pp 421-425, 1981.

27. Laver WG, Air GM, Webster RG. Structure and variation of antigenic sites on influenza virus hemagglutinin. In: Nayak DP, Fox CF, eds. Genetic variation among influenza viruses: ICN-UCLA symposia on molecular and cellular biology. Vol 22. New York: Academic Press, pp 283-296, 1981.

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74. Chambers TM, Webster RG. Modulation of highly pathogenic avian influenza with defective interfering particles. Proceeding of the 3rd International Symposium - Avian Influenza, pp 218-226, The University of Wisconsin, Madison, May 27-29, 1992.

75. Klimov AI, Markushin SG, Prosch S, Sokolov NI, Heider H, Heider AM, Ginzburg VP, Gorodkova NV, Gambaryan AS, Matrosovich MN, Cox NJ, Webster RG. Some aspects of using antiviral compounds in the treatment and control of influenza virus infection. Proceeding of the 3rd International Symposium on Avian Influenza, pp 354-367, May 27-29, 1992.

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91. Webster RG, Fedson DS. Lessons for the future: Pandemic influenza. In Severe Acute Respiratory Syndrome, In press, 2004.

OTHER PROFESSIONAL ACTIVITIES:

Editorial Board Member: Infection and Immunity, Experimental Cell Biology
Editorial Board Member: Problems of Virologi

NATIONAL COMMITTEES:

Associate Coordinator, US-USSR Joint Committee for Health Cooperation on "The Ecology of Human Influenza and Animal Influenza Related to Human Infection"

Collaborator, World Health Organization, Study of Influenza Viruses in Animals

INSTITUTIONAL COMMITTEES:

1974	Credentials-Tenure Committee
1975-78	Ad Hoc Committee on Containment Facilities (Chairman)
1980	Sabbatical Leave Committee
1981	Animal Facilities Care Committee
1984-85	Credentials-Tenure Committee (Chairman 1985)
1988 to present	Executive Committee

INVITED PRESENTATIONS:

1969	International Conference of Hong Kong Influenza, Atlanta, Georgia
1969	Symposium on the Biology of Large RNA Viruses, Cambridge, England
1970	International Congress for Microbiology, Mexico City, Mexico
1971	International Congress for Virology, Budapest, Hungary
1971	American Society for Microbiology, Kentucky-Tennessee Branch, Bowling Green, Kentucky
1971	Workshop on Influenza: Immunologic Methodology and Surveillance, Atlanta, Georgia
1971	Workshop on Influenza Virus Polypeptides and Antigens, Madison, Wisconsin
1972	World Health Organization Symposium on Ecology of Influenza Viruses, Moscow, USSR
1972	Workshop on Epidemiology of Influenza, Washington, DC
1972	Workshop on Influenza Vaccines for Men and Horses, London, England
1973	ICN-UCLA Molecular Biology Conference: Symposium on Virus Research, Squaw Valley, California
1973	International Symposium on Comparative Immunodiagnosis of Viral Infections, Mont Gabriel, Canada

1973 Symposium on Acute Respiratory Diseases, Walter Reed Army Institute of Research, Washington, DC
1973 Workshop VI. Animal Influenza: Its Significance to Human Infection (sponsored by NIAID), Madison, Wisconsin
1973 World Health Organization Meeting On Animal Influenza, Geneva, Switzerland
1974 Symposium on Virus Antibody Interaction, Leeds, England
1975 Pacific Science Congress (13th), Vancouver, Canada
1976 International Conference on Equine Infectious Diseases, Lyon, France
1976 Working Group on Pandemic Influenza, Rougemont, Switzerland
1977 American Society for Microbiology, Conference on Myxoviruses, Tampa, Florida
1977 Conference on Hemagglutinin Subunits, Sandoz Institute, Vienna, Austria
1977 GO Broun Symposium on Influenza Viruses, St. Louis, Missouri
1977 International Association of Biological Standardization, Symposium on Influenza Immunization, Geneva, Switzerland
1977 International Symposium on Microbial Ecology, Otago University, Dunedin, New Zealand
1977 World Health Organization, Consultation on the Ecology of Influenza, Geneva, Switzerland
1978 Fogarty International Center and World Health Organization, International Meeting on the Ecology of Influenza, Bethesda, Maryland
1978 Influenza Workshop, Australian National University, Canberra, Australia
1978 International Congress for Virology (IV), The Hague, The Netherlands
1979 American Society for Microbiology, Kentucky-Tennessee Branch Meeting, Memphis, Tennessee
1979 International Workshop on Structure and Variation in Influenza Virus, Australian National University, Canberra, Australia
1979 National Institute of Health, Influenza B Meeting, Bethesda, Maryland
1979 New York Academy of Science, Conference on Genetic Variation of Viruses, New York, New York
1979 Pacific Science Congress (XIV) (Sponsored by US-USSR Science Exchange Program), Khabarovsk, USSR
1979 Royal Society of the United Kingdom, London, England
1979 World Health Organization Meeting on the Ecology of Influenza Viruses, London, England
1979 Workshop on the Structure of the Influenza Virus Hemagglutinin, Australian National University, Canberra, Australia
1980 Conference on Ecology of Influenza Viruses, Boston, Massachusetts
1980 Conference on New and Useful Techniques in Rapid Viral Diagnosis, National Institutes of Health, Bethesda, Maryland
1980 US-USSR Symposium on Influenza and Acute Respiratory Disease, Alma-Ata, USSR
1980 World Health Organization Consultation on Influenza A Virus Nomenclature, Geneva, Switzerland
1980 World Health Organization Consultation on the Ecology of Influenza Viruses, Munich, West Germany
1981 American Throacic Association Meeting, Detroit, Michigan
1981 Australian National University International Plant Virology Meeting, Kialoa Field Laboratory, Kialoa, New South Wales, Australia
1981 Australian National University, School of Biological Sciences, Canberra, Australia
1981 ICN-UCLA Symposium on Genetic Variation Among Influenza Viruses, Salt Lake City, Utah
1981 Ortho Pharmaceutical, Ltd. and Ortho Diagnostics Symposium on Diagnostic and Therapeutic Applications of Monoclonal Antibodies in Infectious Diseases, Don Mills, Ontario, Canada
1981 Symposia on Microbiology, Munich, Germany
1982 Conference on Influenza: B-cell or T-cell Immunity, Oxford University, Oxford, England
1982 Workshop on the Origin of Pandemic Influenza Viruses, Beijing, Peoples' Republic of China
1982 World Health and Organization Meeting on Ecology of Influenza Viruses, London, England
1983 Pacific Science Congress (XV), Dunedin, New Zealand
1983 Annual Conference on Protein Structure and Function (VIII), Lorne, Australia
1983 American Society for Virology Annual Meeting, East Lansing, Michigan
1983 Beecham Colloquium on the Molecular Virology and Epidemiology of Influenza, London, England

1983 National Congress of Microbiology (IX), Valladolid, Spain
1984 USDA Meeting on Avian Influenza Virus, Hyattsville, Maryland
1984 USDA Meeting on H5N2 Influenza Virus in Chickens, Hyattsville, Maryland
1984 Experimental Virology Study Section, Bethesda, Maryland
1984 USDA Meeting on Avian Influenza Virus, Lancaster, Pennsylvania
1984 Seminar at University of Mississippi, Oxford, Mississippi
1984 Australia and New Zealand Association for the Academy of Science Meeting, seminar in Sydney, Australia, and consultation in Canberra, Australia
1984 USDA Meeting on Avian Influenza Viruses in Chickens, Hyattsville, Maryland
1984 NIH Study Section, Bethesda, Maryland
1984 North Central Avian Disease Conference, Sioux Falls, South Dakota
1984 Avian Influenza Symposium, University Park, Pennsylvania
1984 Workshop on Cell Substrates for Influenza Virus, Bethesda, Maryland
1984 Third Annual ASV Meeting, Madison, Wisconsin Visited Influenza Labs, Hong Kong, Japan
1984 International Symposium of Influenza Virus Hemagglutinin, Osaka, Japan
1984 Sixth International Congress of Virology, Sendai, Japan
1984 Technical Consultants for Avian Influenza Meeting, Hyattsville, Maryland
1984 Seminar at University of Tennessee, Knoxville, Tennessee
1984 Lecture to Wildlife Committee at US Animal Health Association Meeting, Fort Worth, Texas
1984 Workshop entitled "Status of Atenuated Influenza Vaccines," Bethesda, Maryland
1985 Seminar on Influenza, Vanderbilt University, Nashville, Tennessee
1985 Consultation on Human and Animal Influenza Viruses with World Health Organization Officials, Geneva, Switzerland
1985 Seminar at George Washington University, St. Louis, Missouri
1985 Thirty-Sixth Annual Southern Conference on Avian Diseases at the University of Georgia Center for Continued Education, Athens, Georgia
1985 Cold Spring Harbor Meeting on Influenza as Antigens, Cold Spring Harbor, New York
1985 "Law Lecture" at Cornell University, Ithaca, New York
1985 Seminar on Influenza, Lancaster, Pennsylvania
1985 Immunology Symposium, Kalamazoo, Michigan
1985 NIH Contract Review, Bethesda, Maryland
1985 Seminar to Becton-Dickinson Company, Raleigh-Durham, North Carolina
1985 Seminar on H5N2 Avian Flu, Lafayette, Indiana
1985 UCLA Symposia on Options for the Control of Influenza, Keystone, Colorado
1985 International Conference on Antivirals, Bath, England, Joint Study on Host Range Variants of Influenza, London, England
1985 Lecture and Ph.D. Committee Meeting at University of Alabama, Birmingham, Alabama
1985 Scientific Meeting at DI Ivanovsky Institute, Moscow, USSR
1985 Biology of Negative Strand Viruses Meeting, Cambridge, England
1985 Lecture to Delmarva Poultry Conference, Ocean City, Maryland
1986 Antarctic Research Program on Influenza, Antarctica
1986 Study on Influenza in Live Poultry Market, New York, New York
1986 US Animal Health Meeting, Louisville, Kentucky
1986 World Health Organization, Consultation on Influenza in Lower Animals, Geneva, Switzerland
1986 Modern Approaches to New Vaccines Meeting, Cold Spring Harbor, New York
1986 Second International Symposium on Avian Influenza, Athens, Georgia
1986 International Meeting on Virology, Giessen, West Germany
1986 Subcommittee on Reevaluation or Nomenclature of Avian Influenza, St. Louis, Missouri
1987 USA/USSR Influenza Virus Exchange Program, Leningrad and Moscow, USSR
1987 Sixth Annual Meeting of American Society for Virology, Chapel Hill, North Carolina
1987 NIH Meeting on Influenza Viruses, Bethesda, Maryland
1987 VII International Congress of Virology, Edmonton, Alberta, Canada
1987 National Task Force on Influenza in Wildlife and Their Impact on Domestic Poultry, Madison, Wisconsin
1987 U.S. Animal Health Meeting, Salt Lake City, Utah
1987 Guest Lecturer, University of Minnesota, Minneapolis, Minnesota
1987 National Academy of Sciences Meeting on Vaccines to AIDS, Washington, DC

1988 Seventh International Conference on Negative Strand Viruses Meeting, Dinard, France
1988 First Asia-Pacific Congress of Medical Virology, Singapore
1989 101st Meeting of the National Advisory Allergy and Infectious Diseases Council, Bethesda, Maryland
1989 International Workshop on the Use of X-Ray Crystallography in the Design of Antiviral Agents, Kona, Hawaii
1989 Seminar at Wayne State University, Detroit, Michigan
1989 Seminar at Ohio State University, Columbus, Ohio
1989 Seminar at East Carolina State University, Kinston, North Carolina
1989 Anti-Infective Conference, Scottsdale, Arizona
1989 Conference on Emerging Viruses: Evolution of Viruses and Viral Diseases, New York, New York
1989 Seminar at Louisiana State University, Shreveport, Louisiana
1989 Seminar at National Institutes of Health, Tokyo, Japan
1989 Southern Association for Clinical Microbiology, Memphis, Tennessee
1989 Scientific Basis of Medicine Seminar Series, Worcester, Massachusetts
1989 Live Attenuated Influenza Virus Vaccine Meeting, Alexandria, Virginia
1990 Society for General Microbiology at the University of Swansea, South Wales, United Kingdom
1990 Seminar at Univeristy of Alabama-Birmingham, Birmingham, Alabama
1990 UCLA Symposium, Keystone, Colorado
1990 Seminar at University of Michigan, Ann Arbor, Michigan
1990 SmithKline Beecham Pharmaceuticals Anti-infective Conference, Palm Springs, California
1990 Virology Symposium at the Annual Meeting of the Canadian Society for Microbiology, Calgary, Canada
1990 VIIIth International Congress of Virology, Berlin, Germany
1990 Seminar at University of Nebraska-Lincoln, Lincoln, Nebraska
1991 Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut
1991 WHO/OIE Meeting: Host Cell Selection of Influenza Virus Variants, England
1991 Workshop on Acute Respiratory Infections, New Delhi, India
1991 Second Asia-Pacific Congress of Medical Virology, Bangkok, Thailand
1991 Fundacion Juan March, Madrid, Spain
1992 WHO/OIE Influenza Meeting, Newmarket, England
1992 Third International Symposium on Avian Influenza, Madison, Wisconsin
1992 SmithKline Beecham Pharmaceuticals Anti-infective Conference, Tucson, Arizona
1992 100 Years of Virology, Leningrad, Russia
1992 Options for the Control of Influenza II, Courchevel, France
1992 Merck Sharp & Dohme Research Laboratories, Westpoint, Pennsylvania
1992 Burroughs Wellcome Symposium, Research Triangle Park, North Carolina
1993 First International Workshop on Viral and Bacterial Diseases, Memphis, Tennessee
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JOURNAL OF VIROLOGY, Nov. 1999, p. 9679–9682
0022-538X/99/$04.00+0

Vol. 73, No. 11

Rescue of Influenza A Virus from Recombinant DNA

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Received 13 July 1999/Accepted 5 August 1999

We have rescued influenza A virus by transfection of 12 plasmids into Vero cells. The eight individual negative-sense genomic viral RNAs were transcribed from plasmids containing human RNA polymerase I promoter and hepatitis delta virus ribozyme sequences. The three influenza virus polymerase proteins and the nucleoprotein were expressed from protein expression plasmids. This plasmid-based reverse genetics technique facilitates the generation of recombinant influenza viruses containing specific mutations in their genes.

Reverse genetics for negative-strand RNA viruses, first developed for influenza virus (8, 22), has dramatically changed our understanding of the replication cycles of these viruses. In addition, this methodology has allowed genetic manipulation of viral genomes in order to generate new viruses, which can be used as live, attenuated vaccines or vectors to express heterologous proteins (12). The past 5 years have witnessed the rescue of most of the important nonsegmented, negative-strand RNA viruses from recombinant DNA. First, Schnell et al. (30) succeeded in the recovery of rabies virus from cloned DNA. Shortly after, rescue systems were developed for vesicular stomatitis virus (21, 32), respiratory syncytial virus (5, 18a), measles virus (29), Sendai virus (14, 20), and more recently for human parainfluenza type 3 (7, 17), rinderpest virus (1), simian virus 5 (16), bovine respiratory syncytial virus (4), and Newcastle disease virus (27). Bridgen and Elliott (3) succeeded in rescuing a segmented, negative-strand RNA virus, a bunyavirus, from cDNA. In general, all these methods rely on intracellular reconstitution of ribonucleoprotein (RNP) complexes from RNA and viral proteins, i.e., nucleoprotein and RNA-dependent RNA polymerase, which are introduced into cells by a variety of techniques. Generally, a recombinant vaccinia virus expressing T7 RNA polymerase is used to drive transcription of antigenomic positive-sense RNA as the template in order to initiate the replication cycle. Alternatively, transcription is driven by a T7 RNA polymerase, which is constitutively expressed in specific cell lines. Successful recoveries have also been reported by directly transfecting naked RNA (plus sense or minus sense) into cells expressing the essential proteins for encapsidation, transcription, and replication (20). Although reverse genetics techniques allowing genetic manipulation of negative-strand RNA viruses were established for influenza A virus before other negative-strand RNA viruses (8, 22, 31), full recovery of infectious influenza virus from cDNA without the use of helper virus has proved to be technically more difficult.

The genome of influenza A virus consists of eight segments of single-stranded, negative-sense RNA (25). The minimal set of viral proteins required for encapsidation, transcription, and replication of the viral genome are the three subunits of the viral RNA-dependent RNA polymerase complex (PB1, PB2, and PA) and the nucleoprotein (NP) (18). Initially, in order to manipulate the genome of influenza virus, RNPs were reconstituted in vitro from RNA transcribed from plasmid DNA in the presence of polymerase proteins and NP isolated from purified influenza virus (8, 9). The in vitro-reconstituted RNPs were transfected into cells infected with a helper influenza virus, which provided the remaining viral proteins and RNA segments, resulting in the generation of transfectant viruses. This technique has been extremely useful in advancing our understanding of the molecular biology and pathogenicity of influenza viruses. However, it relies on highly specialized selection methods to isolate the transfectant viruses from the helper virus, which restricts its use to certain RNA segments of a limited number of viral strains.

More recently, alternative methods for introducing influenza virus RNPs into cells have been developed, based on intracellular reconstitution of RNPs from in vivo-transcribed RNA and intracellularly expressed viral proteins (23, 24, 28, 33). We showed that the three polymerase proteins (PB1, PB2, and PA) and the nucleoprotein (NP) expressed from recombinant plasmids could encapsidate, transcribe, and replicate an influenza virus viral RNA (vRNA)-like RNA containing a chloramphenicol acetyltransferase (CAT) reporter gene in transfected human 293 cells (28). This vRNA-like reporter gene was introduced into the cells by transfection of a plasmid DNA (pPOLI-CAT-RT) with a truncated human RNA polymerase I (Pol I) promoter (nucleotides [nt] −250 to −1) positioned upstream of the vRNA-coding region. The sequence of the hepatitis delta virus genomic ribozyme was positioned downstream of the vRNA-coding region in order to ensure that RNA processing gave the correct 3′ end of the vRNA. It has also been demonstrated that, by replacing the plasmid coding for the CAT reporter gene with a plasmid encoding an authentic influenza vRNA segment, intracellularly reconstituted RNP complexes could be rescued into transfectant viruses upon infection of the transfected cells with an influenza helper virus. Thus, helper virus-based rescue systems using the RNA Pol I promoter-driven reverse genetics technique have been established for the segments encoding the neuraminidase (NA), the hemagglutinin (HA), the NS1 and NEP proteins, and the polymerase 2 basic protein (PB2) (11, 13, 26, 28). These results suggested that coexpression of the eight vRNA segments of influenza virus with the three polymerase proteins and the NP might allow rescue of infectious influenza virus from plasmid DNA.

In this report we describe the rescue of influenza A virus

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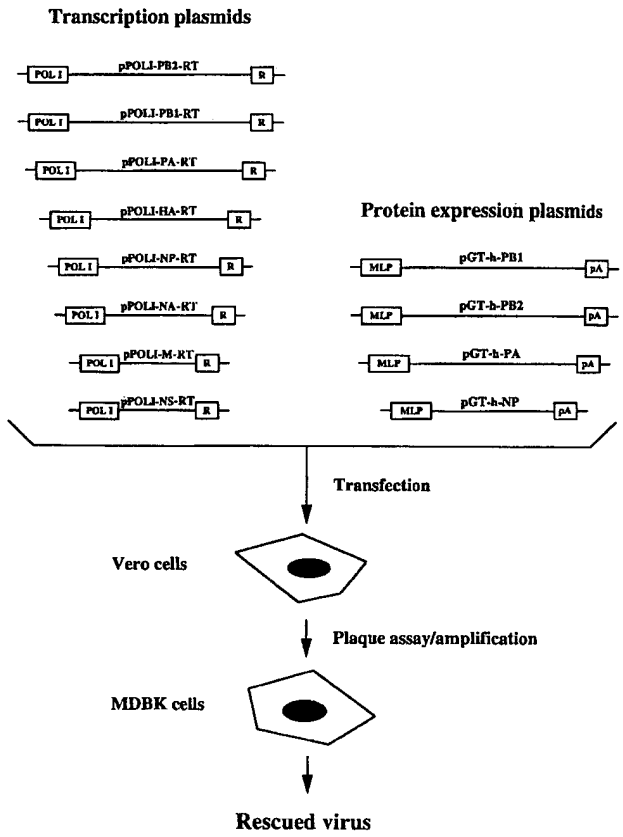

FIG. 1. Schematic representation of the plasmid-based rescue system for influenza A virus. The pGT-h set of protein expression plasmids was constructed by inserting the open reading frames of PB1, PB2, PA, and NP proteins into the *BclI* cloning site of the pGT-h plasmid (2). The PB1 and PA genes were derived from influenza A/WSN/33 virus. The PB2 and NP genes were derived from influenza A/PR/8/34 virus. The viral genomic sequences of influenza A/WSN/33 virus were cloned into pUC18- or pUC19-based plasmids between a truncated human RNA Pol I promoter (nt −250 to −1) (19) and sequences of the hepatitis delta virus ribozyme in an analogous way as described for pPOLI-CAT-RT (28). Genetic tags were inserted into the HA- and NA-encoding plasmids by using conventional mutagenic techniques. For viral rescue, 5 μg of each of the polymerase protein expression plasmids (pGT-h-PB1, pGT-h-PB2, and pGT-h-PA), 10 μg of the NP-expressing pGT-h-NP, and 3 μg of each of the eight vRNA-coding transcription plasmids (pPOLI-PB2-RT, pPOLI-PB1-RT, pPOLI-PA-RT, pPOLI-HA-RT, pPOLI-NP-RT, pPOLI-NA-RT, pPOLI-M-RT, and pPOLI-NS-RT) were diluted to a concentration of 0.1 μg/μl in 20 mM HEPES buffer (pH 7.5). The DNA solution was added to diluted DOTAP liposomal transfection reagent (Boehringer) containing 240 μl of DOTAP and 720 μl of 20 mM HEPES buffer (pH 7.5). The transfection mixture was incubated at room temperature for 15 min, mixed with 6.5 ml of MEM containing 0.5% fetal calf serum, 0.3% bovine serum albumin, penicillin, and streptomycin and added to near-confluent Vero cells washed with phosphate-buffered saline in 8.5-cm dishes (about 10^7 cells). At 24 h after transfection, the transfection mixture was removed and the cells were incubated with 8 ml of fresh medium, which was replaced daily for 4 days. The harvested medium from transfected dishes was screened for rescued influenza virus by plaquing and amplification on MDBK cells. POL I, truncated human RNA polymerase I promoter; R, genomic hepatitis virus ribozyme; MLP, adenovirus type 2 major late promoter; pA, polyadenylation sequence from SV40.

from recombinant DNA. The system is entirely plasmid driven and does not involve the use of any helper or heterologous virus (Fig. 1). In order to recover infectious influenza virus from cloned cDNA, we used a mixture of eight plasmids expressing the individual vRNA segments of influenza A/WSN/33 virus from a truncated human Pol I promoter. We also replaced the previously used four protein expression plasmids, which expressed PB1, PB2, PA, and NP under the control of a mouse hydroxymethylglutaryl-coenzyme A reductase (HMG) promoter (28), with plasmids expressing the same proteins under the control of the adenovirus type 2 major late promoter (pGT-h-PB1, pGT-h-PB2, pGT-h-PA, and pGT-h-NP) (2). Cotransfection of these four plasmids with pPOLI-CAT-RT into 293 or Vero cells resulted in CAT expression (results not shown). We decided to use Vero cells for the virus rescue, since they support the growth of influenza A/WSN/33 virus better than 293 cells (about 1 log difference in maximum viral titers) (results not shown).

For virus rescue, near-confluent Vero cells in 8.5-cm-diameter dishes, were cotransfected with the four protein expression plasmids and the eight vRNA transcription plasmids (pPOLI-PB2-RT, pPOLI-PB1-RT, pPOLI-PA-RT, pPOLI-HA-RT, pPOLI-NP-RT, pPOLI-NA-RT, pPOLI-M-RT, and pPOLI-NS-RT). After 24 h, the transfection medium was removed from the cells and it was replaced with 8 ml of fresh medium (MEM) containing 0.5% fetal calf serum, 0.3% bovine serum albumin, penicillin, and streptomycin. The transfected cells were maintained for at least 4 days after transfection. Every day, the medium from the transfected cells was collected and assayed for the presence of influenza virus by plaquing a 0.5-ml aliquot on MDBK cells by standard methods. The rest of the medium was transferred into 75-cm^2 flasks of subconfluent MDBK cells for amplification of any rescued virus. This procedure resulted in the recovery of infectious influenza virus on day 4 posttransfection. We obtained about 10 to 20 PFU of virus from an 8.5-cm dish containing approximately 10^7 cells. The rescued virus showed a specific property which is characteristic of influenza A/WSN/33 virus, i.e., it formed plaques on MDBK cells in the absence of trypsin. The plaques formed by the rescued virus were comparable in size to those formed by an authentic wild-type influenza A/WSN/33 virus grown on the same MDBK cells (results not shown).

To formally prove that the viral plaques observed on MDBK cells were formed by virus derived from the cloned cDNA, we analyzed two of the eight vRNA segments into which genetic tags were introduced. The HA segment contained a mutation of 6 nucleotides near the 3′ end of the vRNA (26). Nucleotides 31 to 35 from the 3′ end (3′-UUUUG-5′) were replaced with 3′-AAAAC-5′, resulting in an amino acid substitution at amino acid 4 (K→F) and at amino acid 5 (L→V) near the N terminus of HA within the signal peptide. In addition, a silent C→U mutation was created at nucleotide 40. These changes introduced several new restriction sites, including an *SpeI* site. The NA segment contained two silent mutations at nucleotides 1358 and 1360, introducing a novel *SacI* restriction site (28). Medium from MDBK cells infected with the rescued transfectant virus was used to isolate vRNA. Short regions of the HA and NA vRNA containing the genetic tags were amplified by reverse transcription-PCR (RT-PCR) and then analyzed by digestion with *SpeI* and *SacI* restriction enzymes, respectively. As a control, the same regions of the HA and NA segments were amplified from vRNA isolated from authentic A/WSN/33 virus using the same RT-PCR primers. As expected, the PCR products obtained from both viruses had identical sizes (Fig. 2, compare lanes 2 and 5 and lanes 7 and 10). Those originating from the HA and the NA segments of the rescued transfectant virus could be digested with *SpeI* and *SacI*, respectively (lanes 3 and 8). However, the PCR products from the authentic A/WSN/33 virus were, as expected, not digested (lanes 6 and 11). The omission of reverse transcriptase in control RT-PCR reactions resulted in no visible PCR products (lanes 4 and 9).

It should be pointed out that we have succeeded in recovering influenza virus from plasmids expressing negative-sense vRNA. This seems to contradict some earlier studies, which

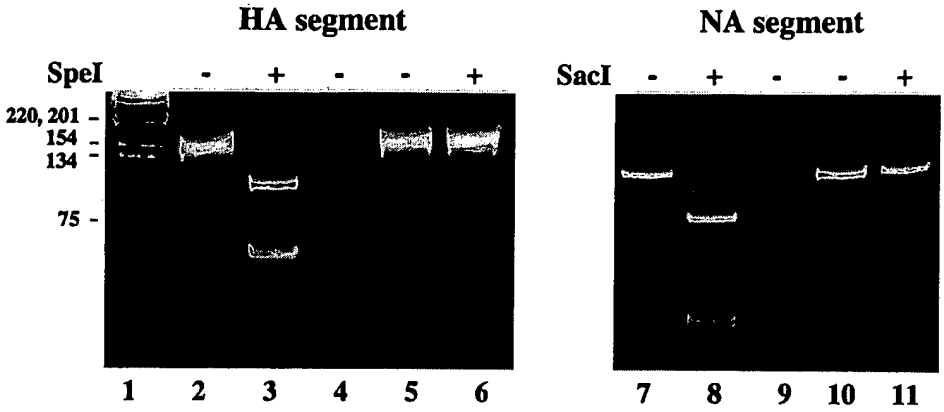

FIG. 2. Demonstration of the presence of genetic tags in the HA and NA vRNA segments of the rescued virus by RT-PCR and restriction enzyme analysis. vRNA of the rescued virus was isolated from medium of infected MDBK cells. One hundred microliters of the medium was treated with 5 U of RNase-free DNase to remove any residual plasmid DNA carried over. After 15 min at 37°C, vRNA was isolated by using the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. vRNA from authentic wild-type A/WSN/33 virus was isolated from purified virus as described previously (10). The first 149 nt at the 3′ end of the HA vRNA were amplified by RT-PCR using oligonucleotide primers 5′-GCGCTCTAGAGCAAAAGCAGGGGAAAATAA-3′ (corresponding to nt 1 to 21) and 5′-CGCGAAGCT TCTCGAATATTGTGTCAAC-3′ (corresponding to nt 129 to 149), resulting in a 165-nt-long PCR product. To amplify the sequence containing the genetic tag from the NA segment, primers 5′-TGGACTAGTGGGAGCATCAT-3′ (corresponding to nt 1280 to 1309) and 5′-GAACAAACTACTTGTCAATGGT-3′ (corresponding to nt 1367 to 1388) were used in RT-PCR to produce a 108-nt PCR product. The HA- and NA-specific PCR products were incubated for 2 h at 37°C in the presence (+) or absence (−) of 10 U of *Spe*I and *Sac*I restriction enzymes, respectively. Samples were analyzed on 16% polyacrylamide gels and stained with ethidium bromide. Lanes: 1, DNA size markers (sizes in nucleotides are indicated); 2, 3, 7, and 8, PCR products from the rescued virus; 4 and 9, control reactions omitting reverse transcriptase; 5, 6, 10, and 11, PCR products from the authentic wild-type A/WSN virus.

emphasized the importance of using positive-strand RNA for rescuing negative-strand RNA viruses (3, 29a, 30). However, more recent successful recoveries of negative-strand RNA viruses from negative-sense RNA have been also reported (7, 20). Since at early stages posttransfection, positive-sense mRNA from the four protein expression plasmids coexists with naked negative-sense genomic vRNA transcribed from the transcription plasmids, inevitably double-stranded RNA can form. Formation of double-stranded RNA could lead to the induction of interferon-mediated antiviral responses and consequently to suppression of the growth of any rescued virus. Therefore the use of a cell line, such as Vero, which is known to be deficient in interferon expression (6), might be an important factor for successful virus rescue. Further work, however, is needed to prove this.

At present, we are able to rescue 1 to 2 infectious viral particles from about 10^6 transfected cells, which corresponds with the "average" recoveries obtained for other negative-strand RNA viruses. By increasing transfection efficiencies and optimizing the ratio of transfected plasmids it might be possible to obtain higher recoveries of virus. Recently, Gómez-Puertas et al. (15) demonstrated that by optimizing plasmid ratios they can significantly increase the formation of influenza virus-like particles from expressed viral proteins. In addition, cell lines expressing essential proteins for encapsidation, transcription, and replication of viral genomic RNA (PB1, PB2, PA, and NP) could help to reduce the number of plasmids needed and thus increase the efficiency of rescue.

In summary, we have rescued a recombinant influenza A virus by cotransfecting eight transcription plasmids for the individual vRNA segments and four protein expression plasmids, entirely from cDNA in the absence of any helper virus. The identity of the rescued virus was confirmed by providing evidence for the presence of two genetic tags in two different genome segments. The development of an entirely plasmid-based rescue system for influenza A virus opens the way for the study of different aspects of influenza virus replication and its interactions with the host cell. In addition, it allows full manipulation of the genome of the virus, which might result in the development of new vaccine strains not only for influenza, but for other infectious agents by introducing specific foreign epitopes into influenza virus proteins. In contrast to the earlier helper virus-based rescue techniques, the plasmid-based system can easily be used for the generation of infectious influenza viruses containing multiple mutations in several different genes at the same time.

We thank Andrew Gannon, Selvon St. Clair, and Stephan Pleschka for constructing several plasmids. We also thank Leo Poon, Jason Paragas, Thomas Zürcher, Masayoshi Enami, and David Pritlove for helpful discussions and Richard Jaskunas for advice.

This work was supported in part by grants from the MRC (programme grant G9523972 to G.G.B.) and the NIH (P.P. and A.G.-S.). E.F. was supported by the Max Kade Foundation in New York (January 1997 to February 1998) and by the MRC programme grant to G.G.B. in Oxford (March 1998 to the present).

ADDENDUM IN PROOF

Following submission of this paper, Neumann et al. (G. Neumann, T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, G. Hobom, and Y. Kawaoka, Proc. Natl. Acad. Sci. USA **96**:9345–9350, 1999) provided independent evidence for a plasmid-based rescue of influenza A virus.

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Efficient generation and growth of influenza virus A/PR/8/34 from eight cDNA fragments

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Available online 22 April 2004

Abstract

A reverse genetics system for the generation of influenza virus A/PR/8/34 (NIBSC vaccine strain) from plasmid DNA was developed. Upon transfection of eight bidirectional transcription plasmids encoding the gene segments of A/PR/8/34 into 293T cells, virus titers in the supernatant were about 10^4 $TCID_{50}$/ml. The production of A/PR/8/34 in 293T cells was compared to that of A/WSN/33, for which virus titers in the supernatant were 10^7–10^8 $TCID_{50}$/ml. Time-course analysis of virus production indicated that the differences in virus titers were due to reinfection of 293T cells by A/WSN/33 but not A/PR/8/34. Indeed, virus titers of A/PR/8/34 comparable to those of A/WSN/33 were achieved upon addition of trypsin to the culture medium of transfected cells. The production of chimeric viruses revealed that the difference in virus titers between A/PR/8/34 and A/WSN/33 are determined primarily by differences in the surface glycoproteins hemagglutinin and neuraminidase and the polymerase protein PB1. In conclusion, high-titer virus stocks of recombinant influenza A/PR/8/34 virus can be produced as well as virus stocks with much lower titers, but without the requirement of virus amplification through replication.

Keywords: Influenza A/PR/8/34; Reverse genetics; Chimeric viruses; Fourth 3' nucleotide

1. Introduction

For a long time the fundamental research of influenza A viruses has been hampered by the lack of availability of efficient reverse genetics systems. Although the earliest reverse genetics techniques for negative stranded RNA viruses were in fact developed for influenza A virus (Enami et al., 1990; Luytjes et al., 1989), the rescue of this virus exclusively from recombinant DNA was achieved only recently (Fodor et al., 1999; Neumann et al., 1999). Recombinant influenza virus was produced upon transfection of eukaryotic cells with a set of eight plasmids from which each of the genomic viral RNA (vRNA) segments was transcribed by RNA polymerase I and a set of four additional plasmids expressing the nucleoprotein (NP) and the polymerase proteins PB1, PB2, and PA. The reported efficiencies of virus production using these 12-plasmid systems were relatively low with less than 10^4 plaque-forming units (pfu) of influenza virus A/WSN/33 per ml of transfected cell supernatant. Neumann et al. reported that upon co-expression of five additional plasmids encoding the hemagglutinin (HA), neuraminidase (NA), matrix proteins 1 and 2 (M1 and M2) and non-structural protein 2 (NS2), virus titers in the supernatants could be increased up to 5×10^7 pfu/ml. An elegant modification of these 12 and 17-plasmid systems came from Hoffmann et al. who implemented bidirectional vectors to reduce the number of transfected plasmids to eight. With this system, the negative-stranded vRNA and the positive-stranded mRNA can be synthesized from the same plasmid and virus titers up to 2×10^7 were reported (Hoffmann et al., 2000). The ability to produce recombinant influenza A virus rapidly and at such high titers will greatly facilitate future influenza virus research. Indeed, several influenza virus strains have now been produced from recombinant DNA to address a number of fundamental research questions in the influenza virus field (Hatta et al., 2001, 2002). In addition, these techniques may be used to produce "conventional" vaccine viruses and to design live attenuated vaccines through genetic engineering. Finally, the use of influenza A viruses as gene delivery vectors and

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doi:10.1016/j.virusres.2004.02.028

to express foreign proteins of interest may now be employed.

It is important to note that the reverse genetics systems described above are all based on influenza virus A/WSN/33 (H1N1). Although influenza virus A/WSN/33 has been used successfully to address many research questions, the surface glycoproteins of this virus have properties that may be undesirable for certain purposes. The NA of A/WSN/33 can bind plasminogen that upon conversion to plasmin can cleave the HA to yield functional HA_1 and HA_2 subunits (Goto and Kawaoka, 1998). As a result, the virus can replicate without trypsin in in vitro cell cultures. For certain purposes, such as mutagenesis studies, virus replication in the transfected producer cells may be undesirable since reverse mutations and second-site mutations may occur. Moreover, for the generation of reassortant viruses to be used as vaccines, influenza virus A/PR/8/34 has been the strain of choice for many years. Therefore, we have designed a reverse genetics system to produce recombinant influenza virus A/PR/8/34. We have used both the 12-plasmid and 8-plasmid systems and compared the virus production of A/PR/8/34 with A/WSN/33. We conclude that virus titers of $\sim 10^4$ can be obtained without virus replication in the transfected cell culture which can be boosted to $>10^7$ when the virus is allowed to replicate. This reverse genetics system may thus be useful for research purposes as well as for the production of vaccine virus.

2. Materials and methods

2.1. Cells and viruses

Madin–Darby Canine kidney (MDCK) cells were cultured in EMEM (BioWhittaker) supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate, 10 mM Hepes and non-essential amino acids. 293T cells were cultured in DMEM (BioWhittaker) supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM sodiumpyruvate, and non-essential amino acids.

Influenza virus A/PR/8/34 was kindly provided by Dr. Wood, the National Institute for Biological Standards and Control, Potters Bar, United Kingdom. Because this strain is adapted for replication in embryonated chicken eggs and may not replicate optimally in mammalian cell cultures, this virus was passaged seven times at a low multiplicity of infection in MDCK cells grown in Episerf media (Gibco BRL) supplemented with 10 IU/ml penicillin and 10 μg/ml streptomycin. After the seventh passage virus titers of 10^8 $TCID_{50}$/ml were obtained routinely.

2.2. Transfection of 293T cells

Transient calcium phosphate-mediated transfections of 293T cells were performed essentially as described (Pear et al., 1993). Cells were plated the day before transfection in gelatinized 100 mm diameter culture dishes to obtain 50% confluent monolayers. After overnight transfection with 25–50 μg plasmid DNA, the transfection medium was replaced with fresh medium supplemented with 2% FCS for virus production or 10% FCS for all other transfections. Cells were incubated for 30–72 h, after which supernatants were harvested and cells were analyzed for fluorescence if appropriate. Plasmid pEGFP-N1 (Clontech, BD Biosciences, Amsterdam, The Netherlands) was transfected in parallel in all experiments and the percentage of fluorescent cells was measured in a FACScan, confirming that the transfection efficiency ranged from 95 to 100%. Virus-containing supernatants were cleared by centrifugation for 10 min at 300 × *g*. Virus titers in the supernatant were determined either directly or upon storage at 4 °C for less than 1 week, or at −80 °C for longer than 1 week.

2.3. Plasmids

Plasmids pPOLI-CAT-RT, pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP were a kind gift from Drs. García-Sastre and Palese (Mount Sinai School of Medicine, New York, USA), plasmids pHL1863 and pHL2428 from Dr. Hobom (University of Giessen, Germany) and plasmids pHW2000, and pHW181 through pHW188 from Dr. Webster (St. Jude Children's Research Hospital, Memphis, TN, USA).

The human RNA polymerase I promoter (Phu) was amplified by PCR using plasmid pHL1863 as template and cloned in plasmid pSP72 (Promega Benelux, Leiden, The Netherlands) using XhoI and XbaI sites included in the primers. The murine RNA polymerase I terminator or the hepatitis delta ribozyme sequence were amplified by PCR using plasmids pHL2428 or pPOLI-CAT-RT as templates and cloned in pSP72-Phu using XbaI and BamHI sites present in the primers to give plasmids pSP72-PhuTmu and pSP72-PhuThep, respectively. Flanking the XbaI site between the Phu and Tmu or Thep sequences we included BpuAI sites to enable the forced directional cloning of influenza A virus cDNAs in these vectors. The eight genomic segments from influenza virus A/PR/8/34 were amplified by RT-PCR and cloned in pSP72-PhuThep (segments 2 and 6) or pSP72-PhuTmu (all other segments). Vector pSP72-PhuThep was used for segments 2 and 6 because pSP72-PhuTmu did not yield the desired recombinant plasmids for unknown reasons. Of note, vectors pSP72-PhuTmu and pSP72-PhuThep work equally well in transient assays in 293T cells (data not shown). To generate bidirectional expression vectors, plasmid pHW2000 was modified so that the BsmBI sites were at the same positions relative to the RNA polymerase I promoter and terminator sequences as the BpuAI sites in our own constructs. The eight genomic segments from influenza virus A/PR/8/34 were subsequently amplified by PCR and cloned in this modified pHW2000 vector. All plasmids were sequenced using a Dyenamic ET

terminator sequencing kit (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and an ABI 373 automatic DNA sequencer (PE Biosystem), according to the instructions of the manufacturer. All PCR primer sequences and plasmid maps are available on request.

2.4. Virus titration

Virus titrations were performed as described previously (Rimmelzwaan et al., 1998). Briefly, 10-fold serial dilutions of the transfected cell supernatants were prepared in infection medium. Infection medium consisted of EMEM (BioWhittaker) supplemented with 4% bovine serum albumine (fraction V, GibcoBRL), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate, 10 mM Hepes, non-essential amino acids, and 25 μg/ml trypsin. Prior to inoculation, the cells were washed twice with PBS. One hundred milliliter of the diluted culture supernatants was used to inoculate a confluent monolayer of MDCK cells in 96 wells plates. After 1 h at 37 °C the cells were washed again with PBS and 200 μl fresh infection medium was added to each well. At 3 days post infection, the supernatants of these cultures were tested for HA activity as an indicator for infection of the cells in individual wells. The titers of infectivity were calculated from 10 replicates according to the method of Spearman–Karber.

3. Results

3.1. A reverse genetics system for influenza virus A/PR/8/34

Influenza virus A/PR/8/34, obtained from the National Institute for Biological Standards and Control, United Kingdom, was passaged seven times in MDCK cells in the presence of trypsin. This virus was found to replicate to high titers in embryonated eggs and MDCK cell cultures (data not shown). The eight genomic segments of this virus were amplified by PCR and cloned into plasmids pSP72-PhuTmu or pSP72-PhuThep. Each of the eight plasmids was sequenced and the sequences were compared with those of A/PR/8/34 and other influenza A virus sequences available from the Influenza Sequence Database (http://www.flu.lanl.gov) (Table 1). For the total genome of A/PR/8/34 which is 13 588 nucleotides in length, we found 111 nucleotide substitutions as compared to sequences of A/PR/8/34 available from the database, resulting in 39 amino acid substitutions. This is not surprising since the passage history of the two A/PR/8/34 strains may be quite different. Analysis of all the sequences available from the Influenza Sequence Database revealed that of these 39 amino acid substitutions only one was unique to A/PR/8/34.

The eight constructs encoding the gene segments of A/PR/8/34 were transfected into 293T cells together with expression plasmids for the polymerase proteins and nucleoprotein of influenza virus A/PR/8/34: HMG-PB2, HMG-PB1, HMG-PA, and HMG-NP (Pleschka et al., 1996). At 72 h after transfection, an infectious virus titer of 1×10^3 $TCID_{50}$/ml of influenza virus A/PR/8/34 was detected in the culture supernatant. However, when this experiment was subsequently repeated five times, virus could be produced only once more with equally low virus titers (data not shown). The inability to generate recombinant virus reproducibly was not due to low transfection efficiencies, since transfections with pEGFP-N1 performed in parallel with these experiments revealed >99% fluorescent cells in these cultures as measured in a FACScan.

In the meantime, a bidirectional 8-plasmid reverse genetics system was described by Hoffmann et al. that we compared with our own 12-plasmid system. To this end, cDNAs encoding the gene segments of influenza virus A/PR/8/34 were cloned into plasmid pHW2000 (Hoffmann et al., 2000).

Table 1
Comparison of nucleotide and amino acid sequences of the genome of MDCK-adapted A/PR/8/34* with those of A/PR/8/34 and A/WSN/33 from the Influenza Sequence Database

Gene segment	Encoded protein	A/PR/8/34			A/WSN/33		
		Accession number	Nucleotide substitutions	Amino acid substitutions	Accession number	Nucleotide substitutions	Amino acid substitutions
1	PB2	NC002023	19	7	J02179	89	26
2	PB1	NC002021	18	8	J02178	62	22
	PB1F2		3	3		10†	8†
3	PA	NC002022	20	1	X17336	65	17
4	HA	NC002017	13	9	J02176	105	54
5	NP	NC002019	15	4	M30746	56	15
6	NA	NC002018	9	5	J02177	82	38
7	M1	NC002016	6	0	L25818	34	5
	M2			2			9
8	NS1	NC002020	11	2	M12597	32	8
	NS2			1			6

* These sequences are available from the Influenza Sequence Database under accession numbers ISDN13419-13426.
† Total insertion counted as a single substitution.

Of note, the sequence of each of the A/PR/8/34 gene segments was identical to that in the 12-plasmid system. Transfection of the eight plasmids encoding the gene segments of influenza virus A/PR/8/34 resulted in a virus titer in the supernatant of ~10^4 $TCID_{50}$/ml 30 h post transfection. More importantly, the virus titers obtained in these transfection experiments were highly reproducible. The titers that we obtained upon transfection of constructs encoding the eight gene segments of influenza virus A/WSN/33 into 293T cells were much higher, however, ranging from 10^7–10^8 $TCID_{50}$/ml.

3.2. Effect of mutations at position 4 of the 3′ terminus

The eight genomic cDNAs inserted in both the 12-plasmid system and the 8-plasmid system were generated using primers specific for the 12 conserved nucleotides (nt) at the 3′ terminus and 13 nt at the 5′ terminus. The virus-specific sequence at the 3′ terminus was UCGUUUUCGUCC, despite the fact that for A/PR/8/34 gene segments 1, 2, 3, 6, and 7 the fourth nucleotide position was reported to be C rather than U (according to the Influenza Sequence Database; http://www.flu.lanl.gov). To test the effect of mutations at the position 4 nt in the 3′ terminus, we generated a set of 8 plasmids containing the eight gene segments of A/PR/8/34 with a C at position 4. The nucleotide sequence of each of these gene segments was identical to the sequence of the original constructs except for this fourth nucleotide. Upon transfection of 8 plasmids with a C at position 4, virus titers were 5.2 × 10^3 $TCID_{50}$/ml, which is slightly higher than upon transfection of 8 plasmids with a U at position 4 (mean virus titer of 3.3 × 10^3 $TCID_{50}$/ml). We next generated sets of recombinant viruses in which each of the genomic segments was replaced with a segment containing C at position 4. These viruses were all produced at comparable levels. The virus titers obtained upon transfection of plasmids representing "wild type" A/PR/8/34 according to the Influenza Sequence Database, with a U in segments 4, 5, and 8 and a C in segments 1, 2, 3, 6, and 7 resulted in a virus titer of 1.1 × 10^4 $TCID_{50}$/ml. From these data we concluded that the low virus titers obtained with A/PR/8/34 were not due to mutations at position 4 in our set of plasmids.

3.3. Increased virus titers due to reinfection

The HA precursor protein (HA_0) of influenza A viruses is cleaved by cellular proteases into HA_1 and HA_2 subunits to yield membrane fusion-competent virus particles. For many in vitro cell cultures infected with influenza A virus, trypsin is added to the culture medium to enable cleavage of HA. Influenza virus A/WSN/33 can replicate in cell cultures without the addition of trypsin to the culture medium. The NA of A/WSN/33 can bind plasminogen that is converted to plasmin, which can subsequently cleave the HA_0 into functional HA_1 and HA_2 subunits (Goto and Kawaoka, 1998). We therefore wished to test if the differences in virus titers

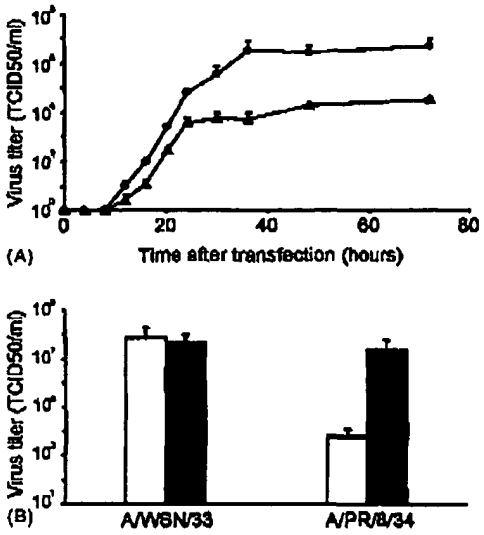

Fig. 1. Virus production in the presence and absence of trypsin. Supernatant of 293T cells transfected with constructs encoding A/WSN/33 (●) or A/PR/8/34 (▲) was harvested at different time points after transfection and titrated on MDCK cells (panel A). Average and standard deviation calculated from two independent experiments are shown. After transfection, trypsin was added to the supernatant of 293T cells transfected with constructs encoding A/PR/8/34 or A/WSN/33 (B). Supernatants were harvested 72 h after transfection and titrated on MDCK cells. (White bars) no trypsin and (black bars) with trypsin. Average and standard deviation calculated from three independent experiments are shown.

between influenza viruses A/WSN/33 and A/PR/8/34 could be explained by trypsin-independent replication in the 293T cells by A/WSN/33 but not by A/PR/8/34.

To this end, we first performed time-course analyses of virus production from 293T cells transfected with constructs encoding either influenza virus A/PR/8/34 or A/WSN/33. Influenza viruses A/PR/8/34 and A/WSN/33 were produced at the same rate during the first 24 h of virus production after transfection. However, from 24 h post transfection onwards, virus production from cells transfected with constructs encoding A/PR/8/34 hardly increased, while virus production from cells transfected with constructs encoding A/WSN/33 continued to increase logarithmically until 36 h post infection (Fig. 1A). The A/WSN/33 virus particles produced late after transfection could well be derived from 293T cells infected with virus produced in the early phase.

To gain further evidence that the high influenza virus A/WSN/33 titer could indeed be caused by the trypsin-independent infection of 293T cells by influenza virus A/WSN/33 but not by A/PR/8/34, we next added trypsin to the culture medium after transfection of 293T cells to a concentration of 0.25 mg/ml. Indeed, upon the addition of trypsin, the virus titers of influenza virus A/PR/8/34 were at the same level as those of influenza virus A/WSN/33 (Fig. 1B). These data indicate that the inability of influenza virus A/PR/8/34 to infect 293T cells without the addition

of trypsin to the culture medium can explain relatively low virus titers of recombinant virus produced from 293T cells. By adding trypsin to the culture medium of transfected 293T cells it is possible to produce high titers of influenza virus A/PR/8/34.

3.4. Analysis of A/WSN/33-A/PR/8/34 chimeric viruses

We next wished to address the question whether NA was the sole determinant of the difference in virus titers obtained with influenza virus A/PR/8/34 and A/WSN/33 due to trypsin-independent replication of the latter virus. To this end, chimeric influenza viruses were produced consisting of seven gene segments of influenza virus A/WSN/33 and one of influenza virus A/PR/8/34 and vice versa and virus titers were determined in MDCK cells. Virus titers obtained upon transfection of 293T cells with constructs encoding seven influenza virus A/PR/8/34 gene segments and one influenza virus A/WSN/33 gene segment were lower than those of wild type influenza virus A/PR/8/34, except when segments 2 (PB1) or 7 (M) of A/WSN/33 were used (Fig. 2A). Although PB1 and M could be partially responsible for low A/PR/8/34 virus titers, the titers obtained with A/PR/8/34 and WSN-PB1 or WSN-M were not nearly as high as wild type A/WSN/33. Therefore, it appeared that none of the gene segments of influenza virus A/PR/8/34 were solely responsible for the low virus titers of influenza virus A/PR/8/34 compared to A/WSN/33.

Viruses with seven influenza virus A/WSN/33 gene segments and one gene segment derived from A/PR/8/34 all yielded titers below wild type A/WSN/33 titers. Upon exchange of gene segments 1, 3, 5, 7, and 8 virus titers were reduced by less than one order of magnitude. The exchange of HA and NA gene segments resulted in a 97-fold and 75-fold reduction in virus production, respectively. Influenza virus A/WSN/33 with PB1 of A/PR/8/34 produced a virus titer that was four orders of magnitude lower than that of wild type A/WSN/33 (Fig. 2B).

Since the receptor-binding activity of HA needs to be balanced by the receptor-removing activity of NA, we generated chimeric virus in which the HA and NA genes of influenza viruses A/PR/8/34 and A/WSN/33 were exchanged simultaneously. Supernatants were harvested 72 h after transfection of 293T cells and titrated on MDCK cells. Compared to 293T cells transfected with constructs encoding all eight influenza virus A/WSN/33 gene segments, cells transfected with constructs encoding six influenza virus A/WSN/33 gene segments and HA and NA of influenza virus A/PR/8/34 produced 141-fold less virus. The 293T cells transfected with six influenza virus A/PR/8/34 gene segments and HA and NA of influenza virus A/WSN/33 produced 224-fold more virus than cells transfected with eight constructs encoding influenza virus A/PR/8/34 (Fig. 3). These chimeric viruses thus demonstrated that the viral surface glycoproteins play a significant role in determining the virus titers produced from 293T cells. Since we already showed that PB1 had a significant influence on virus titers produced in 293T cells (Fig. 2), we next exchanged PB1 together with HA and NA. Recombinant influenza virus A/WSN/33 with segments 2, 4 and 6 of A/PR/8/34 yielded extremely low (34.1 $TCID_{50}$/ml) virus titers from transfected 293T cells. The reciprocal exchange of A/PR/8/34 with segment 2, 4, and 6 of A/WSN/33 yielded virus titers of 3.3×10^6 $TCID_{50}$/ml, which is in the same range as virus titers obtained with wild type influenza virus A/WSN/33. These data suggest that the differences in virus titers between recombinant influenza virus A/WSN/33

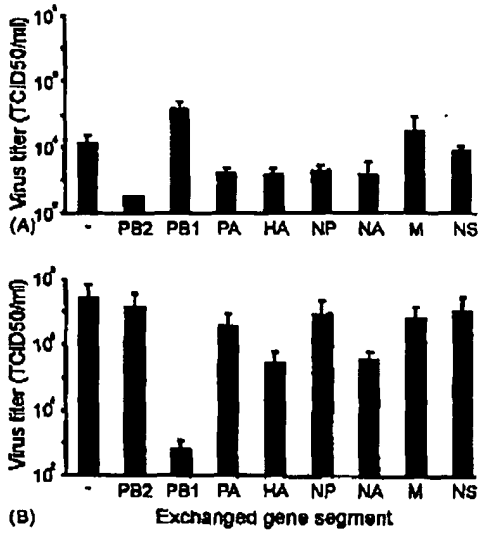

Fig. 2. Production of chimeric influenza viruses from transfected 293T cells. Cells were transfected with seven constructs encoding the gene segments of A/PR/8/34 and one derived from A/WSN/33 as indicated (panel A) or seven gene segments encoding the gene segments of A/WSN/33 and one derived from A/PR/8/34 as indicated (panel B). The first bar in the panel represents wild type A/PR/8/34 (A) or wild type A/WSN/33 (B). Supernatants of transfected cells were harvested 30 h after transfection and titrated on MDCK cells to determine virus titers. Error bars indicate the standard deviation from three independent experiments.

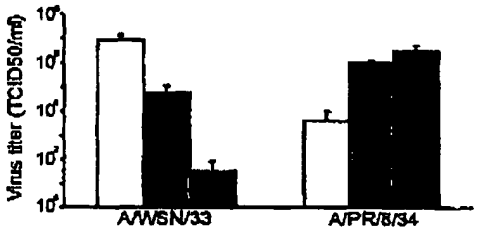

Fig. 3. Virus production upon exchange of HA, NA, and PB1 between A/PR/8/34 and A/WSN/33. Transfected 293T supernatants were harvested at 72 h post transfection and titrated on MDCK cells to determine virus titers. (White bars) wild type virus, (grey bars) exchange of HA and NA, (black bars) exchange of HA, NA, and PB1. Average and standard deviation calculated from four independent experiments are shown.

and A/PR/8/34 are determined primarily by the viral surface glycoproteins and PB1.

4. Discussion

Here, we describe a reverse genetics system for the NIBSC vaccine strain influenza virus A/PR/8/34 adapted to MDCK cells. A bidirectional 8-plasmid transcription system that was first described for influenza virus A/WSN/33 (Hoffmann et al., 2000) was found to be superior to our in-house unidirectional 12-plasmid transcription system. A large difference was observed in the amount of virus produced from 293T cells transfected with constructs encoding influenza virus A/PR/8/34 and A/WSN/33 and the molecular basis for this difference was investigated. The production of A/PR/8/34 could be increased slightly by changing the 3′ position 4 nucleotide from a U to a C in the non-coding region (NCR) of gene segments 1, 2, 3, 6, and 7. The NCRs contain the promoter region for transcription and replication, bind the polymerase proteins and are involved in influenza virus packaging. Lee and Seong showed that the 3′ position 4 nucleotide in the NCR of NA is involved in the temporal regulation of transcription and replication of NA (Lee and Seong, 1998). If this is true for all gene segments, this could explain the fact that the right combination of Us and Cs in all gene segments can lead to a higher virus yield. It should be noted, however, that the differences in virus titers for our mutant viruses were much smaller than expected from these studies on the NA gene segment.

Our time-course analyses showed that despite similar rates of virus production during the early phase after transfection, virus production of A/PR/8/34 and A/WSN/33 was different after >24 h post transfection. Whereas the amount of virus produced by cells transfected with constructs encoding influenza virus A/PR/8/34 hardly increased from 24 h after transfection onwards, the virus titers in the supernatant of cells transfected with constructs encoding influenza virus A/WSN/33 continued to increase logarithmically (Fig. 1A). Presumably, viruses produced relatively early after transfection were the direct result of transfection of the 293T cells whereas the late production of virus may be explained by the amplification of influenza virus A/WSN/33 by infection of 293T cells.

Indeed, the low titers of influenza virus A/PR/8/34 could be explained by the trypsin-dependent replication of this virus in 293T cells. The addition of trypsin to the culture medium of 293T cells transfected with the constructs encoding influenza virus A/PR/8/34 resulted in virus titers in the supernatant similar to those of cells transfected with constructs encoding influenza virus A/WSN/33 (Fig. 1B).

The construction of PB1 chimeric viruses indicated that this gene has a major influence on the amount of virus produced from 293T cells (Fig. 2). The amino acid sequences of PB1 of influenza viruses A/PR/8/34 and A/WSN/33 are 97.0% identical (Table 1). All but one of the amino acid residues that are different between PB1 of A/PR/8/34 and A/WSN/33 are found in other strains for which sequences are available from the Influenza Sequence Database, and it is therefore unlikely that these differences have detrimental effects on virus replication. However, A/PR/8/34 has a unique serine residue at position 394 of PB1, which is in the region of PB1 that is involved in binding to cRNA (Gonzalez and Ortin, 1999). Theoretically, this substitution could be (partially) responsible for the low virus titers obtained with A/PR/8/34. However, mutagenesis of this residue in PB1 of A/PR/8/34 did not result in differences in virus titers (data not shown).

PB1 has polymerase activity and binds to PB2 and PA (Gonzalez et al., 1996), vRNA and cRNA (Gonzalez and Ortin, 1999). One of the possible explanations for the effect of PB1 on virus production is that one of these functions is performed better by PB1 of A/WSN/33 as compared to that of influenza virus A/PR/8/34. Another possibility is that the recently discovered peptide PB1F2, encoded by an alternative open reading frame of PB1 is responsible for differences in virus titers. For influenza virus A/PR/8/34 the PB1F2 open reading frame encodes a 87-residue peptide that causes apoptosis (Chen et al., 2001). In A/WSN/33 this open reading frame is also present but is 3 amino acid residues longer than PB1F2 of A/PR/8/34 and different at seven amino acid positions (Table 1). These differences may affect the function of the protein and thereby have an effect on the amount of virus that is produced. However, this is not very likely since Chen et al. could not detect obvious differences in growth ability in eggs, MDCK or MBDK cells between wild type and PB1F2-deficient viruses.

Upon exchange of the HA and NA of A/PR/8/34 with those of A/WSN/33, virus titers in the 293T supernatant were almost as high as those of wild type influenza virus A/WSN/33. When A/WSN/33 had the HA and NA of influenza virus A/PR/8/34 inserted, virus titers dropped but were not as low as those of wild type A/PR/8/34. Upon exchange of PB1 in addition to HA and NA, virus titers were similar to those of wild type viruses. The effect of HA and NA on virus titers is not determined by WSN-NA alone, since WSN-NA in the context of A/PR/8/34 virus did not result in virus titers similar to A/WSN/33 wild type virus. It was shown by Kawaoka et al. that in the 1957 and 1968 pandemic viruses besides HA and NA, PB1 originated from an avian influenza strain (Kawaoka et al., 1989). Also, Hatta et al. were not able to generate reassortant viruses consisting of seven gene segments of A/Mallard/New York/6750/78 and either PB1, PA, HA or NA of A/Memphis/8/88 (Hatta et al., 2002). These data may suggest that PB1, HA and NA can not be easily exchanged between different influenza virus strains.

Our observation that influenza virus A/WSN/33 replicates in cell culture after transfection indicates that care must be taken if this strain is used for mutagenesis studies. Although high virus yields are beneficial for such experiments, undesired mutations may be acquired during these limited

rounds of virus replication. Similarly, cocultivation of transfected 293T cells with MDCK cells or the addition of trypsin to the culture medium may result in increased virus titers, but could also result in reverse mutations and second-site mutations which are undesirable in many studies. Influenza viruses A/Teal/HK/W312/97 (Hoffmann et al., 2000), A/Hong Kong/483/97, and A/HK/486/97 (Hatta et al., 2001), A/Mallard/NY/6750/78 A/Memphis/8/88 (Hatta et al., 2002) were also generated using a 8-plasmid or 12-plasmid reverse genetics system. For efficient generation of high virus stocks of these viruses, however, it was necessary to amplify these viruses in either MDCK cells or embryonated chicken eggs. Recently Schickli et al. and Hoffmann et al. also produced A/PR/8/34 from recombinant DNA using a 12-plasmid and a 8-plasmid system, respectively. Influenza virus A/PR/8/34 reassortant viruses were produced with HA and NA of different influenza A virus subtypes in 293T cells cocultured with MDCK cells (Hoffmann et al., 2002; Schickli et al., 2001). Using a similar approach we have generated A/PR/8/34(NIBSC)-H3N2 reassortant viruses (data not shown), that could be used to generate vaccine strains in the future.

In the reverse genetics system shown here for the NIBSC vaccine strain of influenza virus A/PR/8/34, replication does not appear to occur in 293T cells and it may therefore be the system of choice for some research projects despite the lower virus titers. When needed, e.g. for vaccine virus production, high virus titers of influenza virus A/PR/8/34 can be achieved by adding trypsin to the culture medium of transfected cells, or cocultivation of transfected 293T cells with cells that are more susceptible to virus replication as was also done by Schickli et al. and Hoffmann et al. (Hoffmann et al., 2002).

Acknowledgements

We wish to thank S. Herfst for excellent technical assistance, J. Wood for providing influenza virus A/PR/8/34, A. Garcia-Sastre and P. Palese for plasmids pPOLI-CAT-RT, pHMG-PB1, pHMG-PB2, pHMG-PA and pHMG-NP, G. Hobom for plasmids pHL1863 and pHL2428, and R. Webster for plasmids pHW2000 and pHW181 through pHW188. Part of this work was supported by Solvay Pharmaceuticals B.V., Weesp, The Netherlands and the Foundation for Respiratory Virus Infections. R.F. is a fellow of the Royal Dutch Academy of Arts and Sciences.

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Vaccine 20 (2002) 3165–3170

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Eight-plasmid system for rapid generation of influenza virus vaccines

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Received 9 January 2002; received in revised form 28 March 2002; accepted 9 May 2002

Abstract

The antigenic variation of influenza A virus hemagglutinin (HA) and neuraminidase (NA) glycoproteins requires frequent changes in vaccine formulation. The classical method of creating influenza virus seed strains for vaccine production is to generate 6 + 2 reassortants that contain six genes from a high-yield virus, such as A/PR/8/34 (H1N1) and the *HA* and *NA* genes of the circulating strains. The techniques currently used are time-consuming because of the selection process required to isolate the reassortant virus. We generated the high-yield virus A/PR/8/34 (H1N1) entirely from eight plasmids. Its growth phenotype in embryonated chicken eggs was equivalent to that of the wild-type virus. By using this DNA-based cotransfection technique, we generated 6 + 2 reassortants that had the antigenic determinants of the influenza virus strains A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), A/teal/HK/W312 (H6N1), and A/quail/HK/G1/97 (H9N2). Our findings demonstrate that the eight-plasmid system allows the rapid and reproducible generation of reassortant influenza A viruses for use in the manufacture of vaccines.

Keywords: Influenza; Vaccines; Reverse genetics

1. Introduction

During the 20th century, influenza A viruses of the H1N1, H2N2, and H3N2 antigenic subtypes have caused epidemics of respiratory disease in humans. In 1997, H5N1 viruses of avian origin caused human illness and death in China [1,2]. In 1998 and 1999, H9N2 viruses circulating in poultry caused disease in humans [3]. Sequence analysis of the *HA* gene of the human isolate A/HK/1073/99 (H9N2) revealed the virus to be closely related to a virus isolated from quail, A/quail/HK/G1/97 (H9N2) [4]. These influenza outbreaks raised concerns about newly emerging influenza A viruses and the possibility of a new pandemic [5]. At the time of the outbreaks, no vaccines were available for protection against the H5 subtype.

The licensed influenza vaccines in current use are inactivated virus vaccines created by growing virus in embryonated chicken eggs and subsequently purifying and inactivating them by chemical means. Each year, the World Health Organization selects subtypes that are representative of strains currently circulating in humans. The efficacy of vaccines requires that the selected vaccine strains be sufficiently closely related to the circulating strains to ensure the induction of effective neutralizing antibodies. However, not all viruses that are closely related are suitable for vaccine production; some grow poorly in eggs. Therefore, the current practice is to generate a high-growth reassortant that combines the high virus yield of the laboratory strain A/PR/8/34 (H1N1) with expression of the glycoproteins of the currently circulating strain [6].

Coinfection with two influenza viruses containing eight segments can theoretically result in the generation of $2^8 - 2 = 254$ different progeny viruses. The selection procedure required to obtain the desired reassortant virus and to verify its gene constellation is cumbersome and time-consuming [7]. Although the reverse genetics method in which cells are transfected with in vitro-generated ribonucleoproteins, reduces the possible number of progeny viruses, a more efficient selection method is needed [8]. Plasmid-driven synthesis of viral RNA and proteins allows the recovery of infectious influenza virus without the need for helper virus infection [9–11]. Therefore, we investigated whether the eight-plasmid system which we established [11] allows the generation of 6+2 reassortant viruses. Here, we demonstrate the utility of this approach for the generation of high-yield reassortants. Our findings suggest that vaccine manufacturers can apply this DNA transfection method to cell lines

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0264-410X/02/$ – see front matter
PII: S0264-410X(02)00268-2

Table 1
Generation of 6 + 2 reassortant influenza A viruses

HA/NA-subtype	Parent virus	HA plasmid	NA plasmid	Recombinant virus[a]
H1N1	A/New Caledonia/20/99 (H1N1)	pHW454-HA	pHW456-NA	rgPR8-H1N1
H3N2	A/Panama/2007/99 (H3N2)	pHW444-HA	pHW446-NA	rgPR8-H3N2
H5N1	A/Goose/HK/437-4/99 (H5N1)	pHW251-HA	pHW246-NA	rgPR8-H5ΔN1[b]
H6N1	A/teal/HK/W312/97 (H6N1)	pHW244-HA	pHW246-NA	rgPR8-H6N1
H9N2	A/quail/HK/G1/97 (H9N2)	pHW409-HA	pHW422-NA	rgPR8-H9N2

[a] Viruses were generated by transfecting cocultured 293T–MDCK cells with six plasmids carrying the six internal genes of the virus strain A/PR/8/34 (H1N1) and two plasmids encoding the desired HA and NA subtypes.
[b] The N1 neuraminidase was derived from A/teal/HK/W312/97 (H6N1).

which are approved for vaccine production for rapid and reproducible generation of influenza virus vaccines.

2. Materials and methods

2.1. Virus strains

The influenza A viruses used in this study (Table 1) were obtained from the repository of St. Jude Children's Research Hospital and were propagated in embryonated chicken eggs.

2.2. RT-PCR and construction of plasmids

RT-PCR was performed with segment-specific primers as described elsewhere [12]. Briefly, RNA was isolated by using the RNeasy kit (Qiagen). RNA was transcribed to cDNA by using Uni12-primer (AGC AAA AGC AGG) and the cDNA was then amplified by using segment-specific primers. The *HA*, *NP*, *NA*, *M*, and *NS* genes of A/PR/8/34 (H1N1) (PR8 virus) were cloned by digesting the PCR fragments with *Bsm*BI or *Bsa*I and ligating them into the cloning vector pHW2000. The *P* genes were cloned by isolating two (PB2, PA) or three (PB1) fragments, digesting them, and ligating them into pHW2000-*Bsm*BI. To ensure that the genes were free of unwanted mutations, the inserted viral cDNAs were sequenced. The eight plasmids containing the full-length cDNA of PR8 virus were designated pHW191-PB2, pHW192-PB1, pHW193-PA, pHW194-HA, pHW195-NP, pHW196-NA, pHW197-M, and pHW198-NS. PCR with the primer pair Bm-HA1 (TAT TCG TCT CAG GGA GCA AAA GCA GGG G) and Bm-NS-890R (ATA TCG TCT CGT ATT AGT AGA AAC AAG GGT GTT TT) was used to clone *HA* genes of several subtypes (nucleotides representing the influenza A virus non-coding regions are underlined). Those primers were also used to characterize the recombinant viruses by RT-PCR. The plasmid pHW251-HA encoding a deletion mutant of the A/Goose/HK/437-4/99 (H5N1) hemagglutinin was derived by PCR amplification of two fragments of the plasmid pHW250-HA encoding the full-length H5. The fragments were digested with *Bsm*BI and inserted into pHW2000-*Bsm*BI. For cloning of pHW409-HA, the H9-PCR fragment was first inserted into the pCR2.1 vector (invitrogen); the resultant plasmid containing the *H9* gene was used to subclone the *H9* gene into pHW2000-*Bsm*BI. The construction of the plasmids pHW244-HA and pHW246-NA representing the *HA* and *NA* genes from A/teal/HK/W312 (H6N1) has been described elsewhere [11]. The Center for Biotechnology at St. Jude Children's Research Hospital determined the sequence of template DNA by using Rhodamine or dRhodamine dye-terminator cycle sequencing ready reaction kits with AmpliTaq® DNA polymerase FS (Perkin-Elmer Applied Biosystems Inc., Foster City, CA) and synthetic oligonucleotides. Samples were separated by electrophoresis and analyzed on PE/ABI model 373, model 373 Stretch, or model 377 DNA sequencers.

2.3. Generation of recombinant viruses

Recombinant viruses were generated by DNA transfection as described previously [11]. Briefly, the day before transfection 293T and MDCK cells were trypsinized, $(0.2–1) \times 10^6$ of each cell line were used for the transfection experiments. Different dilutions ($1:10^3$–$1:10^5$) of virus stocks containing 320–5120 hemagglutination units (HAU) per ml were used to infect the allantoic cavity of 10-day-old embryonated chicken eggs. The data in Table 2 represent results from two or three experiments.

After 48 h, the allantoic fluid was harvested for analysis. H5N1, H6N1, and H9N2 viruses were grown in BL3 facilities at St. Jude Children's Research Hospital.

2.4. Hemagglutination (HA) and hemagglutination inhibition (HI) assays

Fifty microliters of 0.5% chicken red blood cell suspension in PBS was added to 50 μl of two-fold dilutions of virus in phosphate buffered saline (PBS), and the mixture was incubated at room temperature for 30 min. The HA titre was calculated as the reciprocal value of the highest virus dilution that caused complete hemagglutination.

For HI assays, 50 μl of receptor destroying enzyme treated antiserum was titrated, and 25 μl of an equivalent amount of virus (four hemagglutinating doses) was added to each well. After incubation at room temperature for 30 min, 50 μl of a

Table 2
Growth of 6 + 2 reassortant influenza A viruses

Virus	HA titre[a]										
	1	2	3	4	5	6	7	8	9	10	Mean
wtPR8	10240	5120	10240	5120	10240	Dead	10240	10240	5120	10240	8533
rgPR8	10240	20480	320	10240	5120	640	5120	5120	2560	5120	6496
rgPR8-H1N1	2560	1280	1280	2560	2560	640	2560	5120	1280	1280	2112
rgPR8-H3N2	1280	1280	1280	1280	640	2560	2560	2560	1280	640	1536
rgPR8-H5ΔN1	160	320	160	160	320	160	160	320	320	160	224
rgPR8-H6N1	2560	5120	2560	1280	1280	1280	1280	1280	1280	2560	2048
rgPR8-H9N2	5120	5120	10240	5120	5120	5120	5120	10240	5120	5120	6144

[a] The HA titre of the allantoic fluid was determined 48 h after infection of 10-day-old embryonated chicken eggs. Assays were performed with 0.5% chicken red blood cells. Values represent the titres after infection of 10 different eggs.

0.5% suspension of chicken red blood cells was added. The HI titre was determined after 30 min as the reciprocal of the serum dilution that inhibited hemagglutination.

3. Results

3.1. Generation of A/PR/8/34 (H1N1) from eight plasmids

The influenza A virus A/PR/8/34 (H1N1) is well adapted to growth in embryonated chicken eggs and is currently used as the master strain for the production of inactivated vaccines. To generate A/PR/8/34 (H1N1) virus from plasmids, we amplified the eight viral RNA segments by RT-PCR and cloned the fragments into the plasmid pHW2000 [11]. Recombinant viruses were generated by transfection of cocultured 293T–MDCK cells with the resultant eight plasmids (pHW191-PB2, pHW192-PB1, pHW193-PA, pHW194-HA, pHW195-NP, pHW196-NA, pHW197-M, and pHW198-NS). The yield of virus recovered after 72 h was determined to be 2×10^6 pfu/ml by titration of the cell culture supernatant in MDCK cells. The supernatant of cocultured Vero-MDCK cells contained 1×10^4 pfu/ml.

To compare the growth of the wild-type virus (wtPR8) with that of the recombinant virus (rgPR8) generated by reverse genetics, we infected embryonated chicken eggs with wild-type or recombinant virus. The allantoic fluid of 10 infected eggs was harvested 48 h after infection. The virus yield was determined by HA assay. Although the HA titres differed among individual eggs, both viruses had HA titres between 5120 and 10,240 in most of the eggs and were therefore high-yielding isolates (Table 2). These results show that the A/PR/8/34 (H1N1) rgPR8 virus is generated efficiently and reliably from eight plasmids and that the plasmid-derived recombinant virus has the same high-yield phenotype as the wild-type virus.

3.2. Generation of the H3N2 and H1N1 6 + 2 reassortants recommended for human vaccine strains

The A/Panama/2007/99 (H3N2) and A/New Caledonia/20/99 (H1N1) virus strains were recommended by World Health Organization for use in influenza vaccine in the year 2001/2002. To test the utility of the eight-plasmid system to generate reassortants representing these strains, we cotransfected 293T–MDCK cells with plasmids encoding the glycoproteins of A/Panama/2007/99 (H3N2) or A/New Caledonia/20/99 (H1N1) and with the six plasmids encoding the internal genes of A/PR/8/34 (Fig. 1). We found the HA titres in the majority of infected eggs to be 1280–2560 (Table 2). The recombinant rgPR8-H1N1 and rgPR8-H3N2 viruses were shown by HI assay (Table 3) to be antigenically identical to the parental viruses A/Panama/2007/99 (H3N2) and A/New Caledonia/20/99 (H1N1).

3.3. Generation of 6 + 2 reassortants from viruses circulating in Southeast China

The pathogenic H5N1 viruses isolated in 1997 in Hong Kong were hypothesized to have been generated by reassort-

Table 3
Antigenic characterization of wild-type and recombinant viruses by hemagglutination inhibition (HI) assay

Virus	Antiserum	
	Specific[a]	α-H7[b]
wtPR8	2560	<[c]
rgPR8	640	<
A/New Caledonia/20/99 (H1N1)	>5120	80
rgPR8-H1N1	>5120	160
A/Panama/2007/99 (H3N2)	>5120	<
rgPR8-H3N2	>5120	<
A/Goose/HK/437-4/99 (H5N1)	2560	<
rgPR8-H5ΔN1	>5120	<
A/teal/HK/W312/97 (H6N1)	320	<
rgPR8-H6N1	80	<
A/quail/HK/G1/97 (H9N2)	1280	<
rgPR8-H9N2	1280	<
A/Eq/Prague	<	640

[a] Specific antiserum against wild-type virus was used for each subtype.
[b] A/equine/Prague/1/56 (H7N7)-specific antiserum was used as a control.
[c] <: No detectable inhibition of hemagglutination.

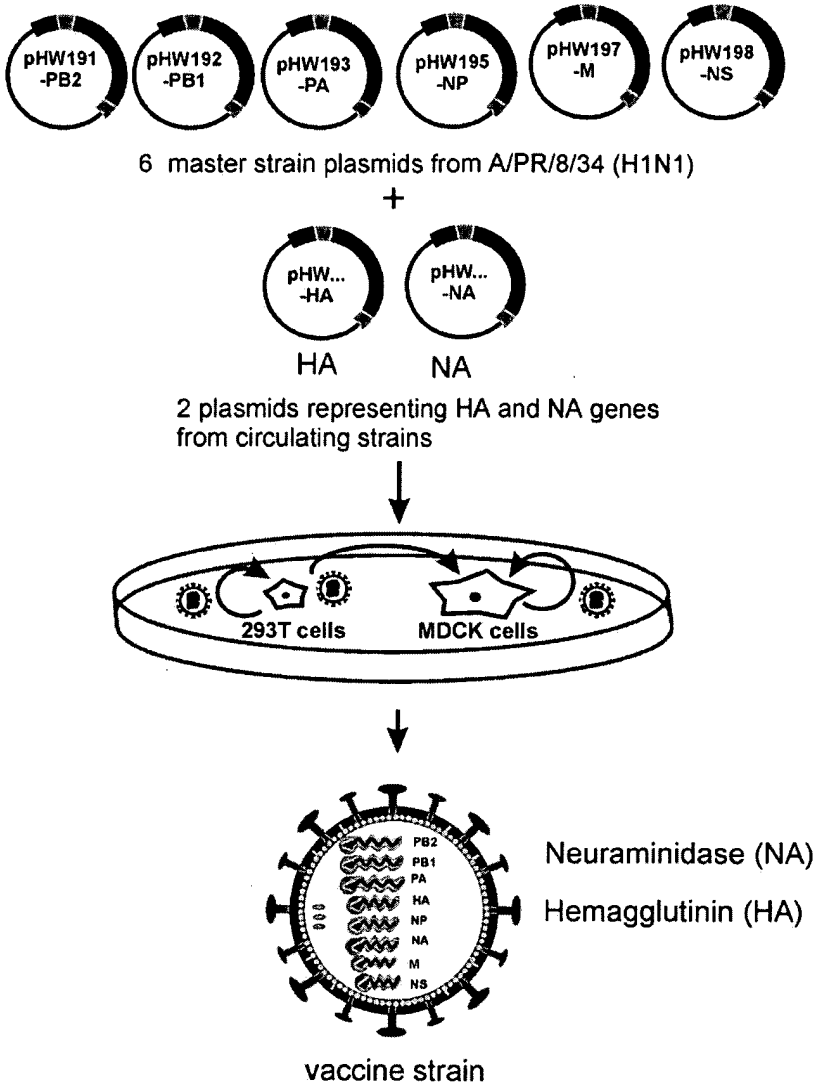

Fig. 1. Eight-plasmid system for rapid generation of reassortant influenza A virus. To test the utility of the eight-plasmid system for generation of reassortant viruses, 293T–MDCK cells were cotransfected with six plasmids representing the A/PR/8/34 (H1N1) master strain and two plasmids representing the desired HA and/or NA subtypes. Influenza viruses were generated within 2–3 days after transfection. Because the viruses were derived entirely from DNA, no selection system was needed to isolate the desired reassortant. The rescued viruses can be used as seed viruses for growth in embryonated chicken eggs.

ment of a Goose-H5 precursor virus [13]. The high homology of six segments of A/quail/HK/G1/97 (H9N2) and seven segments of A/teal/HK/W312/97 (H6N1) to those of the H5N1 viruses suggested that the H5N1 viruses were derived from H9N2 or H6N1 viruses circulating in Asia [4,14,15]. The close relationship of those viruses to the pathogenic H5N1 viruses which caused disease in humans indicates that those viruses could be transmitted to mammalian species. Indeed, H9N2 viruses closely related to the G1 lineage have been isolated from humans [3,16].

To evaluate whether 6 + 2 reassortants could be derived from currently circulating H6N1 and H9N2 subtypes, we cotransfected 293T–MDCK cells with plasmids containing the hemagglutinin and neuraminidase genes from A/teal/HK/W312/97 (H6N1) or A/quail/HK/G1/97 (H9N2) and with the six plasmids representing the internal genes of the PR8 virus. The HA titres of the reassortants were 5120–10,240 for rgPR8-H9N2 and 1280–5120 for rgPR8-H6N1 (Table 2). Antigenic analysis (Table 3) confirmed that the rgPR8-H6N1 and rgPR8-H9N2 viruses were of the same subtype as A/teal/HK/W312/97 (H6N1) and A/quail/HK/G1/97 (H9N2).

Preparation of high-growth reassortants for the production of H5N1 vaccine strains is difficult, because the infected chicken embryos die within 1 day after infection. The high lethality of H5N1 strains in chickens is associated with the basic amino acids of the connecting peptide between the HA1 and HA2 subunits. Therefore, we constructed a plasmid in which the region encoding the connecting peptide (PQ*RERRRKKR* ↓ G) of the H5

from A/Goose/HK/437-4/99 (H5N1) was replaced with a sequence found in the H6 of A/teal/HK/W312 (H6N1) (PQ*IETR* ↓ G). This plasmid and the plasmid encoding N1 neuraminidase from A/teal/HK/W312/97 (H6N1) (closely related to the neuraminidase of the pathogenic H5N1 viruses from 1997) together with the six PR8-plasmids were co-transfected into 293T–MDCK cells resulting in the generation of rgPR8-H5ΔN1 virus. This reassortant virus yielded an HA titre of 160–320, 48 h after infection (Table 2).

3.4. Characterization of the reassortant viruses by RT-PCR

Although controls were included in the experiments and no wild-type viruses were used during the DNA transfection experiments to minimize the possibility of laboratory contaminations, RT-PCR was performed to confirm that the recovered influenza viruses were reassortants with the PR8 backbone. The *NS* and *HA* genes of the 6 + 2 reassortants were amplified by RT-PCR with the primer pair Bm-HA1 and Bm-NS-890R, which were previously shown to amplify these genes [12]. Partial sequencing of the PCR products with segment-specific primers confirmed that the *HA* genes were derived from the designated subtypes and that all of the *NS* genes were derived from PR8 virus. These results show that reassortant influenza A viruses of the H1N1, H3N2, H5N1, H6N1, and H9N2 subtypes can be generated rapidly and reproducibly.

4. Discussion

The eight-plasmid virus generation system that uses the human RNA polymerase-I promoter requires the use of cell lines derived from humans or monkeys because of the species specificity of pol I–mediated transcription [17]. In this study, we used 293T cells for virus generation because those cells have high transfection efficiency resulting in high virus yield. To ensure the safety of a vaccine, cell lines approved for influenza virus vaccines, such as Vero monkey kidney cells can be employed for primary virus generation by DNA transfection. In addition to the use of approved cell lines certified cell culture media and transfection reagents approved for vaccine production have to be used. It is anticipated that the resultant 6 + 2 reassortant viruses after those technical modifications will have the same high-growth phenotype as those generated in this study. For the subsequent production of vaccines, embryonated chicken eggs or continuous cell lines can be used. Because of the limited availability of eggs for the production of virus, continuous cell lines are considered an attractive alternative. There is evidence that human viruses propagated in cell lines are more likely than those propagated in eggs to have an HA that resembles the HA of the original human isolate [18–20]. For the production of vaccines, any cell line which allows the efficient replication of influenza virus, such as Vero cells or St. Jude porcine lung (SJPL) cells, can be used [20–22].

We have demonstrated that H3N2 and H1N1 reassortants generated by DNA transfection grow to moderate to high titres in eggs—a growth level equivalent to that observed in classical reassortment techniques. The H6N1 and H9N2 reassortants from subtypes currently circulating in Southeast China grew to high titres in eggs, indicating that our 6 + 2 approach can generate high-yield reassortants of these subtypes. Our findings support the view that the six internal genes of the PR8 virus are important for the high-yield phenotype. Because rgPR8-H5ΔN1 and rgPR8-H6N1 viruses differed only in the *HA* gene, the lower yield of rgPR8-H5ΔN1 virus was caused by the H5Δ hemagglutinin. These results show that not only the internal genes but also the hemagglutinin gene are determinants of the virus yield in eggs. Deletion of basic amino acids (PQ*RERRRKKR* ↓ G → PQ*RETR* ↓ G) of A/Hong Kong/483/97 (H5N1) resulted in a variant that was attenuated in mice [23]. Possibly, the genetic alteration of the H5 molecule reduces the replication efficiency of a virus in mice and in eggs. Further studies using the eight-plasmid system allows to elucidate whether the yield of H5N1 viruses can be increased by genetic manipulation of the H5 (e.g. the addition of amino acids to the connecting peptide) or the *PR8*-genes.

The use of 6 + 2 plasmids to generate 6 + 2 reassortant influenza A viruses eliminates the need for a selection system, thus simplifying the creation of viruses of the desired subtypes. The rapid generation of reassortants and the improved full-length amplification of influenza gene segments by RT-PCR [12] suggest that plasmid collections representing different subtypes of circulating strains, including those isolated from humans and animals, should be created in concert with surveillance studies. We have rescued reassortant PR8 viruses representing 15 HA and nine NA subtypes (data not shown) by using the same technique; therefore, this method is applicable to all influenza virus subtypes. If new pathogenic viruses should emerge, plasmids representing the closest antigenic subtype can be used to produce virus seed suitable for manufacturing a vaccine.

The classical reassortment method currently in use requires lengthy screening and selection procedures that create a 2–3-month lag time between identification of a new strain and the start of vaccine production. If plasmid collections were available, only 1–3 weeks would be needed to generate reassortants by the DNA transfection method. The vaccines could then enter production and become available within about 4–5 months. This 2-month reduction in lag time could be crucial in reducing the spread of a newly emerging pathogenic virus. Further, generation of 6 + 2 viruses by the PR8-plasmid method does not require multiple passages in eggs, as does the classical reassortment method. Viral adaptation to growth in eggs can alter the HA antigens, thereby compromising the protective effect of the vaccine product [24]. Therefore, the *de novo* generation of reassortant influenza viruses by DNA transfection would produce virus seed more quickly, and the product would be more

reproducible and more likely to closely match the circulating strain antigenically.

Acknowledgements

These studies were supported by Public Health Research Grants AI08831, AI95357 and AI29680 from the National Institute of Allergy and Infectious Diseases, by Cancer Center Support CORE Grant CA-21765, and by the American Lebanese Syrian Associated Charities. We thank Alan Hay for providing the influenza viruses A/Panama/2007/99 (H3N2) and A/New Caledonia/20/99 (H1N1). The excellent technical assistance of Ashley Baker, David Walker, and Nannan Zhou is gratefully acknowledged. We thank Sharon Naron for scientific editing.

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Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines

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Summary

Background In response to the emergence of severe infection capable of rapid global spread, WHO will issue a pandemic alert. Such alerts are rare; however, on Feb 19, 2003, a pandemic alert was issued in response to human infections caused by an avian H5N1 influenza virus, A/Hong Kong/213/03. H5N1 had been noted once before in human beings in 1997 and killed a third (6/18) of infected people.[1,2] The 2003 variant seemed to have been transmitted directly from birds to human beings and caused fatal pneumonia in one of two infected individuals. Candidate vaccines were sought, but no avirulent viruses antigenically similar to the pathogen were available, and the isolate killed embryonated chicken eggs. Since traditional strategies of vaccine production were not viable, we sought to produce a candidate reference virus using reverse genetics.

Methods We removed the polybasic aminoacids that are associated with high virulence from the haemagglutinin cleavage site of A/Hong Kong/213/03 using influenza reverse genetics techniques. A reference vaccine virus was then produced on an A/Puerto Rico/8/34 (PR8) backbone on WHO-approved Vero cells. We assessed this reference virus for pathogenicity in in-vivo and in-vitro assays.

Findings A reference vaccine virus was produced in Good Manufacturing Practice (GMP)-grade facilities in less than 4 weeks from the time of virus isolation. This virus proved to be non-pathogenic in chickens and ferrets and was shown to be stable after multiple passages in embryonated chicken eggs.

Interpretation The ability to produce a candidate reference virus in such a short period of time sets a new standard for rapid response to emerging infectious disease threats and clearly shows the usefulness of reverse genetics for influenza vaccine development. The same technologies and procedures are currently being used to create reference vaccine viruses against the 2004 H5N1 viruses circulating in Asia.

Lancet 2004; **363:** 1099–103

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Introduction

In February, 2003, two family members were admitted to intensive care wards in Hong Kong Special Administrative Region with influenza-like respiratory illness. Avian-like H5N1 influenza viruses were isolated from both patients, one of whom succumbed to infection. This was the first time since 1997 that H5N1 viruses had been identified in human beings, and WHO responded by issuing a pandemic alert. Candidate vaccines were immediately sought. The recent outbreak of severe acute respiratory syndrome (SARS) had been a striking example of the rapid and global spread of an emerging infectious disease. However, even the effects of SARS could be dwarfed by those that could arise with the emergence of an influenza pandemic.

Infection caused by the influenza A virus is a zoonosis, and the animal reservoir of this virus is the aquatic bird populations of the world. The compelling epidemiological link between the presence of the virus in poultry in live-bird markets and the appearance of H5N1 in human beings in 1997 suggested that influenza A viruses can be transmitted directly from avian species to man and can cause severe respiratory disease.[1–3] Although control of the 1997 outbreak was achieved by culling millions of birds in the Hong Kong markets,[4] this episode demonstrated that the capability for an effective global response to emerging influenza threats was poor because of technical, legislative, and infrastructural limitations. A disturbing finding that emerged from this event was that the scientific community was unable to produce an effective vaccine even after several years.

The inactivated human influenza vaccines in use today are derived from essentially modified viruses. By exploiting the segmented nature of the influenza A genome, vaccine manufacturers and the laboratories of the WHO influenza network have produced a reassortant virus carrying the circulating virus's gene segments that encode haemagglutinin and neuraminidase, the major targets of neutralising antibodies. The remaining six-gene segments are supplied from PR8, a laboratory-adapted avirulent H1N1 strain.[5] The resulting reassortant virus has the antigenic properties of the circulating strain and the safety and high-yield properties of PR8.

The first batch of inactivated material against the 1997 H5N1 virus was not ready for clinical trial until 7 months after the second case of human infection arose, and even today the effectiveness of vaccine against this virus has not been proven.[6] A key reason for this delay in the production of an H5N1-specific vaccine was the nature of the virus itself. The H5N1 virus is highly pathogenic in human beings and poultry. The agent must be handled only under conditions of at least biosafety level 3 (BSL3), and it can kill fertilised chicken eggs, the standard medium for the reassortment and

propagation of influenza virus before its inactivation and formulation for use in vaccines. These same traits are present in the 2003 H5N1 virus.

The pathogenic nature of these H5N1 viruses is linked to the presence of additional basic residues in haemagglutinin at the site of cleavage, a step required for haemagglutinin activation and, thus, for virus entry into cells.[7-9] To overcome the high pathogenicity of the virus, polybasic aminoacids have to be eliminated. A rapid, reproducible system to achieve these modifications—ie, plasmid-based reverse genetics—has been developed only in the past 4–5 years[10-12] The potential benefits of reverse genetics for the generation and attenuation of vaccine candidates against highly pathogenic and low pathogenic influenza viruses are enormous.[13-15] However, the host specificity of the RNA polymerase I promoter used in the influenza reverse-genetics systems and the required use of an approved cell line limits the practical options for the system's use in the manufacture of human vaccines. The vaccine-candidate reference virus stock described in this report has been produced entirely on a cell substrate licensed for the manufacture of human vaccine, and as such, is—to our knowledge—the first reverse genetically derived influenza vaccine suitable for testing in clinical trials. We describe the construction of a vaccine reference virus in Good Manufacturing Practice (GMP)-grade facilities in less than 4 weeks from the time of virus isolation. Our findings highlight the speed with which new technologies can be implemented in response to influenza pandemic alerts.

Methods

Cells and A/Puerto Rico/8/34 plasmids

We obtained WHO-approved Vero cells (WHO-Vero, X38, p134) from the American Type Culture Collection (Manassas, Virginia, USA). Passage-142 cells (five passages since their removal from a working cell bank) were used for the rescue of the vaccine-candidate virus. The plasmids containing the genes from PR8 have been described elsewhere.[13]

Virus propagation, RNA extraction, PCR amplification, and haemagglutinin and neuraminidase gene cloning

We obtained A/Hong Kong/213/03 (H5N1) that had been passaged in eggs from the WHO influenza network. The virus was isolated and propagated in 10-day-old embryonated chicken eggs. Total RNA was extracted from infected allantoic fluid with use of the RNeasy kit (Qiagen, Valencia, CA, USA) in accordance with manufacturer's instructions. Reverse transcription was carried out with the uni12 primer (5′-AGCA AAAGCAGG-3′) and AMV reverse transcriptase (Roche, Indiana Biochemicals Indianapolis, USA). The removal of the connecting peptide of the haemagglutinin was done with use of PCR with the following primer sets: (1) Bm-HA-1 (5′-TATTCGTCTCAGGGAGCAA AAGCAGGGG-3′) and 739ΔR (5′-TAATCGTC TCGTTTCAATTTGAGGGCTATTTCTGAGCC-3′); and (2) 739ΔF (5′-TAATCGTCTCTGAAA CTAGAGGATTATTTGGAGCTATAGC-3′) and Bm-NS-890r (5′-ATATCGTCTCGTATTAGTAG AAACAAGGGTGTTTT-3′). We amplified the neuraminidase gene of A/Hong Kong/213/03 using the primer pair Ba-NA-1 (5′-TATTGGTCTC AGGGAGCAAAAGCAGGAGT-3′) and Ba-NA-1413r (5′-ATATGGTCTCGTATTAGTAGAAACAAG GAGTTTTTT-3′). PCR products were purified and cloned into the vector pHW2000 as described previously.[11]

Rescue of virus from Vero cells

The rescue of infectious virus from cloned cDNA was done under GMP conditions. Vero cells were grown to 70% confluency in a 75 cm^2 flask, trypsinised (with trypsin-versene), and resuspended in 10 mL of Opti-MEM I (Invitrogen, Carlsbad CA, USA). To 2 mL of cell suspension we added 20 mL of fresh Opti-MEM I; then, we added 3 mL of this diluted suspension to each well of a six-well tissue culture plate (about 1×10^6 cells per well). The plates were incubated at 37°C overnight. The next day, 1 μg of each plasmid and 16 μL of TransIT LT-1 transfection reagent (Panvera, Madison, WI, USA) were added to Opti-MEM I to a final volume of 200 μL and the mixture incubated at room temperature for 45 min. After incubation, the medium was removed from one well of the six-well plate, 800 μL of Opti-MEM I added to the transfection mix, and this mixture added dropwise to the cells. 6 h later, the DNA-transfection mixture was replaced by Opti-MEM I. 24 h after transfection, 1 mL of Opti-MEM I that contained 1 μg/mL L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemicals, Lakewood, NJ, USA) was added to the cells. About 72 h after the addition of TPCK-trypsin, the culture supernatants were harvested and clarified by low-speed centrifugation; we then injected 100 μL of the clarified supernatant into the allantoic cavity of individual 10-day-old pathogen–free embryonated research grade eggs (Charles River SPAFAS, North Franklin, CT, USA).

Pathogenicity testing in chickens

Ten 4-week-old chickens received intravenous injections of 0·1 mL diluted virus (dilution ratio, 1/10). We monitored chickens for signs of disease for 10 days using the Intravenous Pathogenicity Index, approved by the Office of International Epizooites (OIE). Additionally, we took tracheal and cloacal swabs (in 1 mL of media) 3 days and 5 days after infection, and we did assays for the presence of virus by injection of 0·1 mL into all of three 10-day-old embryonated chicken eggs. Haemagglutination activity in the allantoic fluid of these eggs was assessed after incubation at 35°C for 2 days.

Pathogenicity testing in ferrets

We tested pathogenicity of the vaccine in five young adult male ferrets (Marshall's Farms, North Rose, NY, USA) aged 4–8 months (weight about 1·5 kg) that were shown by haemagglutination inhibition assays to be seronegative for currently circulating human influenza A viruses (H3N2, H1N1) and H5N1 viruses. We anaesthetised the ferrets with inhaled isoflurane, and they were then infected intranasally with 10^6 50% egg infectious dose (EID_{50})/mL of vaccine reassortant virus or wildtype virus. We monitored the ferrets once per day for signs of sneezing, inappetence, and inactivity, and we recorded rectal temperatures and bodyweights. 3, 5, and 7 days after infection, the ferrets were anaesthetised with ketamine (25 mg/kg), and we collected nasal washes using 1 mL of sterile phosphate-buffered saline (PBS) containing antibiotics. We measured titres of virus in these washes with EID_{50} assays.

To further assess the pathogenicity of the viruses, we collected tissue samples from lungs, brain, olfactory bulb, spleen, and intestine for virus isolation and histopathological analysis at the time of death or in the case of three ferrets, after euthanasia at day 3 after infection. The tissues were fixed in 10% neutral buffer formalin, processed and embedded in paraffin, sectioned at 5 μg, stained with haematoxylin and eosin and examined by light microscopy in a blinded fashion.

Stability testing in eggs

To test the stability of the vaccine virus on propagation, we made 16 consecutive passages of the virus in embryonated chicken eggs. A 10^{-4} dilution of the virus was made in PBS, and 0·1 mL of the solution was injected into the allantoic cavities of all of four 10-day-old embryonated chicken eggs. Eggs were incubated at 35°C for 1·5–2 days. After incubation, each egg was candled to determine embryo viability before chilling at 4°C. We harvested 2 mL of allantoic fluid from each egg harvested, and samples were pooled together, tested for haemagglutination activity, and then reinjected into another four eggs.

Role of the funding source

The sponsor had no role in study design, in the collection, analysis, and interpretation of data, in the writing of the report or decision to submit this manuscript for publication.

Results

Alteration of haemagglutinin cleavage site and virus rescue

The first challenge we faced in producing a vaccine against A/Hong Kong/213/03 (H5N1) was to attenuate the virus in preparation for mass production. Previous experiences have shown that removal of the basic aminoacids at the haemagglutinin cleavage site substantially attenuates pathogenic influenza viruses.[15–17] Using a PCR-based mutagenesis approach, we replaced the cleavage site encoded by the haemagglutinin gene of A/Hong Kong/213/03 (H5N1) with that of the avirulent A/teal/Hong Kong/W312/97 (H6N1) (figure 1); this modified haemagglutinin gene and the neuraminidase gene of A/Hong Kong/213/03 (H5N1) were cloned individually into the vector pHW2000.[11] The two resulting plasmids and the six plasmids encoding the remaining proteins of PR8[13] were transfected into WHO-approved Vero cells under GMP conditions to rescue the vaccine seed virus, Δ213/PR8. 36–48 h after transfection, isolated areas of cytopathic effect could be seen on the Vero monolayers. Although addition of further 1 μg aliquots of TPCK-treated trypsin every 24 h led to a proportional increase in the cytopathic effect, it was not required for successful virus rescue. The candidate vaccine strain grew to high titres on subsequent amplification in eggs (haemagglutination titres of 1024–2048) and did not cause embryo death. The vaccine seed virus was unable to form plaques on Madin-Darby canine kidney (MDCK) cells in the absence of trypsin, a trait consistent with that of influenza viruses that lack the polybasic cleavage site, and was antigenically indistinguishable from the parental H5N1 virus in haemagglutination inhibition assays. The rescued virus was fully sequenced and was identical to the plasmids used in its creation.

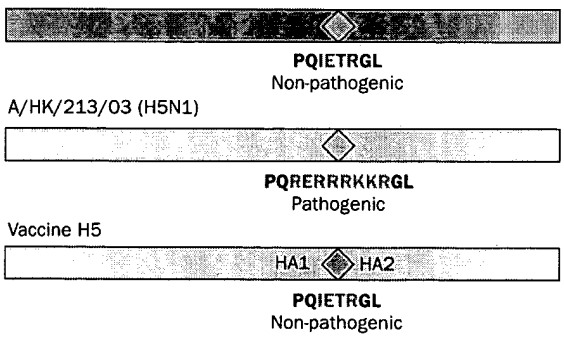

Figure 1: **Creation of haemagglutinin protein of candidate vaccine seed**

Haemagglutinin protein of the candidate vaccine seed (Δ213/PR8) was produced by replacing the connecting peptide of the A/Hong Kong/213/03 haemagglutinin gene with that of the A/Teal/Hong Kong/W312/97 gene.

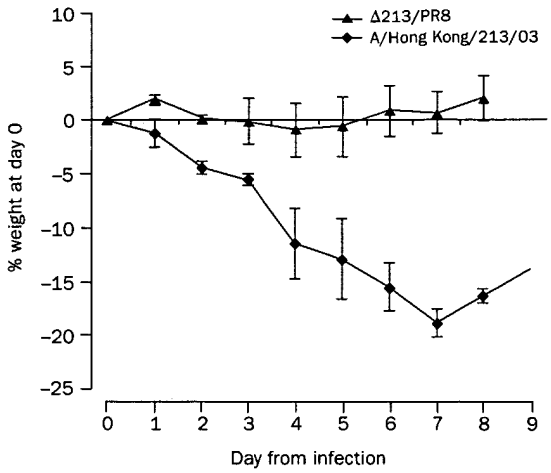

Figure 2: **Weight changes of ferrets infected with wildtype A/Hong Kong/213/03 or Δ213/PR8**

Vertical bars show SD.

Pathogenicity testing of the candidate reference virus

To assess the pathogenicity of the H5N1 vaccine seed virus, we compared the properties of this virus with those of the wildtype A/Hong Kong/213/03 (H5N1) in ferrets and in chickens. By stark contrast with the wildtype virus, which was lethal to all chickens within 48 h of infection, intravenous administration of a 1/10 dilution of Δ213/PR8 did not result in any signs of infection in chickens, and we were unable to detect any virus in swabs of cloacae or tracheae from inoculated birds. Compared with A/Hong Kong/213/03 (H5N1), Δ213/PR8 was attenuated in ferrets that had been inoculated intranasally with 10^6 EID_{50} of virus. Ferrets infected with A/Hong Kong/213/03 had inappetence and weight loss (figure 2), with one infected animal dying 6 days after infection and a second killed 10 days after infection because of hind-limb paralysis. Infection in these animals was characterised by viral shedding until 7 days after infection and replication of virus in the lower respiratory tract and olfactory bulb (as determined by virus isolation). In the A/Hong Kong/213/03 infected animals, there was a mild mononuclear cell infiltrate in the meninges and tracheal submucosal mucous glands and an extensive bronchopneumonia. The pneumatic infiltrate progressed in severity from the bronchi to the pleura. The bronchi and bronchioles contained sloughed necrotic epithelial cells, numerous mononuclear cells, and a few neutrophils. The alveoli were consolidated with inflammatory cells and fibrin (figure 3). By contrast, those ferrets infected with Δ213/PR8 did not lose weight (figure 2) and seemed to remain healthy during the study (14 days) (figure 3). Virus was detected in the nasal washes of these animals at 5 days but not 7 days after infection, and virus was recovered from the upper respiratory tract only. By light microscopy, the meninges and trachea of the Δ213/PR8 infected ferrets did not have an inflammatory infiltrate and only a few neutrophils were noted occasionally in pulmonary bronchi. Our results clearly show that

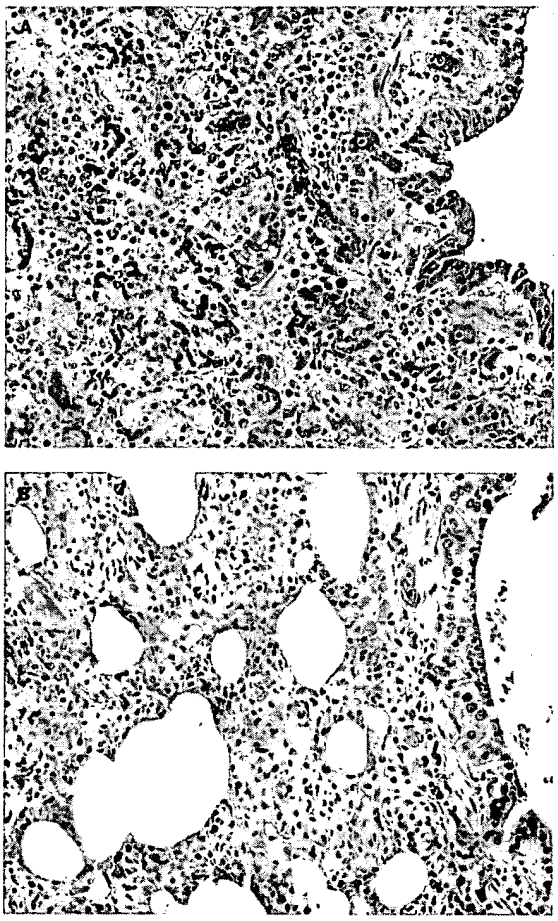

Figure 3: **Ferret lung 3 days after infection with wildtype virus (A) and the reverse genetic virus Δ213/PR8 (B)**
(A) Alveoli are filled with inflammatory cells and the bronchiolar submucosa is oedematous. (B) Alveoli are free of inflammatory cells and there are a few neutrophils on the surface of the bronchiolar epithelium. Magnification ×20.

Δ213/PR8 was attenuated. In view of our findings, this virus can be safely handled with standard precautions in BSL2 containment facilities.

Stability of non-pathogenic phenotype

Because the mechanisms and requirements for the accumulation of basic aminoacids at the haemagglutinin cleavage site are not entirely understood, we wanted to confirm that the altered cleavage site remained stable on multiple passages in embryonated chicken eggs. Such passaging in eggs would occur in transition and amplification of the reference virus to vaccine stock. The rescued virus was stable on continued serial passage in embryonated eggs, and we did not detect any change in nucleotide sequence of the haemagglutinin cleavage site after 16 passages. There was no evidence of changing pathogenicity of the virus and we noted only one dead embryo at passage 15. No haemagglutination activity was evident in this egg and no embryo death was seen in passage 16, which strongly suggests that the death was not related to virus replication. Haemagglutination titres at each passage ranged from 512 to 2048 with no apparent trend of increasing or decreasing titres in subsequent passages.

Discussion

The rapid response in terms of potential vaccine reference virus production to the 2003 H5N1 outbreak differs strikingly from the response to the 1997 episode. This difference is attributable to the new scientific technology available in 2003 and, just as importantly, to the infrastructure for virus surveillance in Hong Kong developed since 1997. The first case of H5N1 influenza in Hong Kong was in May, 1997; yet several months elapsed before this virus was finally characterised as an H5N1 virus. In 2003, the causative agent was identified only hours after admission of the patients to the hospital. The increased awareness, surveillance, and availability of reagents to identify influenza viruses of all subtypes bode well for the rapid identification of viruses that arise from future interspecies transfer events and for the coordination of international vaccine development by WHO. The timely distribution of candidate viruses is a very important step in the development of vaccines for pandemic emergencies. Despite the heightened security and documentation requirements for shipping and receiving potential bioterrorism agents, the H5N1 and SARS outbreaks have shown that in true emergencies, global distribution is feasible.

Although it is pertinent to prepare for future pandemics by stockpiling potential vaccine strains, the H5N1 situation in 2003—and the ongoing H5N1 outbreaks throughout Asia in 2004 (http://www.who.int)—have highlighted the fact that some of the focus of pandemic planning must go into the implementation of technology to rapidly produce vaccines from field isolates. Although viruses similar to A/Hong Kong/213/03 (H5N1) had been circulating in bird populations, these viruses were antigenically distinct, despite high genetic similarities (Guan Y and Peiris JS, unpublished data). That the aminoacid differences are on the globular head of haemagglutinin and seem to be responsible for much of the antigenic difference means that even a vaccine previously prepared from genetically similar precursor viruses might not provide adequate protection. We may well be faced with potential pandemic situations in the future and the rapid production of a matched vaccine will be needed—a point again highlighted by H5N1 outbreaks in 2004. Although the reference virus described in this report was prepared from a virus isolated in a similar geographic region and only a year earlier, it shares only limited antigenic cross-reactivity to the 2004 H5N1 viruses. Hyperimmune sheep serum samples produced against the purified haemagglutinin of Δ213/PR8 has at least a six-fold reduced haemagglutination inhibitory activity against A/Vietnam/1203/04 as compared with A/Hong Kong/213/03. As our findings show, we have the technical capabilities to respond rapidly to outbreaks with a safe and stable reference virus, but there is still much to be accomplished before such viruses can be fully used in pandemic and interpandemic influenza vaccine production.

The use of reverse genetics introduces a number of new processes into influenza vaccine manufacture that are not encountered with standard reassortment methods. One of the most obvious is the need for cultured cells. Although both Vero[18] and MDCK[19,20] cells are in development as substrates for the growth of influenza vaccine, there are additional requirements for the use of cells in reverse genetics. Unfortunately, the number of suitable cell lines is very small. In addition to the regulatory requirements, the choice of cell is also limited by the technology. The plasmid based reverse-genetics systems[10–12] use the species-specific human RNA polymerase I promoter, which

necessitates the use of cells from primate origin. The Vero cell line is probably the only option currently able to meet both regulatory and technical demands. We have shown that Vero cells can be used to successfully rescue H1N1, H3N2, H6N1, and H9N2 viruses on the PR8 backbone using the 8-plasmid system.[21] Others have demonstrated the suitability of Vero cells for alternative influenza virus reverse-genetics systems.[10] Although cultures of Vero cells are easily obtained, only cells from fully tested and licensed cell banks are likely to be acceptable for vaccine manufacture. This issue must be acknowledged and access to such cells must be incorporated as part of future pandemic plans.

That future threats of influenza pandemics will be addressed by the use of the technology described in this report seems inevitable. Despite the presence of low pathogenic surrogate strains, the recent human death from influenza-like illness caused by highly pathogenic H7N7 virus in the Netherlands[22] reinforces the fact that future outbreaks will probably occur in which this reverse-genetics technology provides the logical—and, possibly, the only—way to respond rapidly and effectively. Although our response to the outbreak of H5N1 influenza in 2003 has shown that current scientific capabilities are sufficient to respond to the threat, there are still legal and infrastructural barriers to be overcome.[23] These barriers include licensing and intellectual property issues surrounding what is, essentially, a genetically modified organism. Yet, these difficulties are not insurmountable and pandemic scares such as the 2003 and ongoing 2004 H5N1 outbreaks are forcing commercial and regulatory parties to address these issues with some urgency. With the development of the 2003 H5N1 vaccine reference virus, and ongoing attempts to create the same for the 2004 virus, the challenge in responding to a threat of an influenza pandemic must now be supported by the large-scale manufacture of the vaccine and by clinical trials of a new vaccine manipulated by reverse genetics.

Contributors

R J Webby, D R Perez, J S Coleman, J H Knight, E I Tuomanen, R G Webster designed the study; R J Webby did much of the construction of the vaccine seed virus; D R Perez developed and constructed plasmid templates; Y Guan and J S Peiris characterised and isolated the initial H5N1 virus; J E Rehg participated in the design and analysis of animal safety testing of the candidate H5N1 vaccine seed virus; E A Govorkova participated in the safety testing of the candidate H5N1 vaccine seed virus; L R McClain-Moss participated in the preparation of GMP documentation of the process and was involved in the reconstitution of the vaccine seed virus.

Conflict of interest statement

None declared. The corresponding author has had full access to all the data in the study and has had the final responsibility for the decision to submit this manuscript for publication.

Acknowledgments

We thank Todd Hatchette, Katherine Sturm-Ramirez, and Scott Krauss for expert advice; Ashley Baker, Christie Johnson, Yolanda Sims, Patrick Seiler, Jennifer Humberd, and Kelly Jones for excellent technical assistance; Julia Hurwitz for access to the Vero-cell banks. Editorial assistance was provided by Julia Cay Jones. These studies were supported by grant AI95357 from the National Institute of Allergy and Infectious Disease, by Cancer Center Support (CORE) grant CA21765 from the National Institutes of Health, and by the American Lebanese Syrian Associated Charities (ALSAC).

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Virology 267, 310–317 (2000)
doi:10.1006/viro.1999.0140, available online at http://www.idealibrary.com on IDEAL®

"Ambisense" Approach for the Generation of Influenza A Virus: vRNA and mRNA Synthesis from One Template

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Received September 27, 1999; returned to author for revision December 2, 1999; received December 13, 1999

We present a system for creating influenza virus by generating viral RNA (vRNA) and mRNA from one template. Recently, a system for the generation of influenza A virus entirely from cloned cDNAs was established (Neumann *et al.*, 1999, *Proc. Natl. Acad. Sci. USA* 96, 9345–9350). Cells were transfected with plasmids for RNA polymerase I-driven intracellular synthesis of all eight viral RNAs, and with protein expression plasmids for the synthesis of viral structural proteins. Although this system is highly efficient in virus generation, the construction and cotransfection of 17 plasmids is cumbersome and may limit the use of this system to cell lines that can be transfected with high efficiencies. Synthesizing both vRNA and mRNA from one template would reduce the number of plasmids required for virus generation. Therefore, we generated a bidirectional transcription construct that contains cDNA encoding PB1 flanked by an RNA polymerase I (pol I) promoter for vRNA synthesis and an RNA polymerase II (pol II) promoter for mRNA synthesis. The utility of this approach is proved by the generation of virus after transfecting the pol I/pol II-promoter–PB1 construct together with vRNA- and protein-expression constructs for the remaining seven segments. Because this approach reduces the number of plasmids required for virus generation, it also reduces the work necessary for cloning, probably enhances the efficiency of virus generation, and expands the use of the reverse-genetics system to cell lines for which efficient cotransfection of 17 plasmids cannot be achieved.

INTRODUCTION

Influenza A virus is a negative-strand RNA virus with a segmented genome consisting of eight viral RNA segments. The genomic RNAs contain one or more open reading frames that are flanked by noncoding sequences at the 5′ and 3′ ends of the RNA molecules (Desselberger *et al.*, 1980). The viral RNAs are associated with viral NP and polymerase proteins (PB1, PB2, and PA) in virions and in infected cells to form ribonucleoprotein (RNP) complexes (Hsu *et al.*, 1987). After the RNP complexes enter the nucleus, the RNA segments are replicated and transcribed to yield three types of RNA molecules: The vRNA is transcribed into cRNA, which is copied into vRNA. The vRNA serves as template for the synthesis of mRNA, from which viral proteins are translated. Late in the life cycle, the genomic RNAs and proteins are packaged into new progeny virus particles that can start a new replication cycle.

Several methods have been developed for genetic engineering of influenza A viruses (Luytjes *et al.*, 1989; Zobel *et al.*, 1993). The ribonucleoprotein (RNP) transfection system developed by Palese and coworkers (Luytjes *et al.*, 1989; Enami *et al.*, 1990) is based on the *in vitro* transcription reaction for synthesis of RNA molecules. RNP complexes are made by incubating the RNA transcripts with purified nucleoprotein and the three polymerase subunits (PB1, PB2, PA). After the reconstituted RNP complexes are transfected into eukaryotic cells, a helper virus is used as the source of the proteins needed for replication of the recombinant RNP molecules.

Hobom and coworkers (Zobel *et al.*, 1993; Neumann *et al.*, 1994) developed a system based on the precise transcription of an influenza virus cDNA template by the cellular RNA polymerase I (pol I) complex. In this system, the viral cDNA is inserted between a pol I-promoter and terminator sequence. After *in vivo* synthesis of genomic influenza-like RNA molecules, the nucleoprotein and polymerase proteins are delivered by infection with helper virus, thus forming functional RNPs in mouse or primate cells (Neumann *et al.*, 1994; Hoffmann, 1997). Pleschka and colleagues (1996) also used a pol I-promoter for the transcription of viral cDNA, but the 3′ end of the viral RNA was generated by the autocatalytic cleavage of a hepatitis delta ribozyme sequence. However, the potential use of the RNP- and the DNA-transfection methods is limited, because of the use of a helper virus,

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which requires a strong selection system to obtain the desired virus from a vast background of helper virus.

In a previous study, a plasmid-based system was used to provide both viral RNA and viral proteins for the generation of influenza A viruses without helper virus infection (Neumann *et al.*, 1999). This reverse-genetics system proved to be highly efficient for the generation of the A/WSN/33 (H1N1) virus in 293T cells. However, this system, which contains the pol I-and pol II promoters with the influenza virus cDNAs on different plasmids, requires the construction of at least 12 plasmids for efficient virus recovery. Transfection of cells with this many plasmids may limit the use of this system to cell lines which have a high transfection efficiency. To be able to rescue virus from different cell types may increase the virus yield by enhancing the replication of influenza A virus in these cells and increase the range of cells suitable for the production of vaccines (Govorkova *et al.*, 1996). As a first step in reducing the number of plasmids, we report here the construction and transfection of plasmids containing both the pol I- and pol II-promoter on the same plasmid and present evidence that this system allows the expression of vRNA and protein from one template.

RESULTS AND DISCUSSION

Design and features of the cloning vector pHW12 containing two eukaryotic promoters

Influenza A viruses are segmented viruses that contain RNA molecules with negative-sense polarity. During the replication cycle, recognition of the 5′- and 3′-structures of the eight vRNA segments by the ribonucleoprotein complex proteins (PB2, PB1, PA, NP) results in the replication and transcription of the influenza virus genes. The fact that the terminal sequence elements are highly conserved indicates that a transcribed artificial RNA should have sequences that are the same as those of the 5′ and 3′ ends (Luo *et al.*, 1991; Flick *et al.*, 1996). The cloning vector pHW12 was constructed, allowing the insertion of sequences of interest between the pol I-promoter and terminator by using the restriction endonuclease *Bsm*BI (see Fig. 1A). The pol I-transcription unit is flanked by the pol II-promoter from the cytomegalovirus (CMV) and by the polyadenylation signal of the gene encoding bovine growth hormone. The CMV-promoter, the poly A site, and the backbone of the plasmid are derived from the cloning vector pcDNA3.

PB1 protein expression in the pol I/pol II bidirectional transcription system

To test the pol I/pol II one-plasmid transcription system, we inserted the cDNA of the PB1 gene of A/WSN/33 virus into the cloning vector pHW12 to yield the plasmid pHW52-PB1 (Fig. 1B). *Hin*dIII and *Xho*I restriction sites were inserted into the 5′ and 3′ noncoding regions of gene. These genetic tags were included to ensure the generated recombinant virus could be identified RT-PCR. We expected that human cells transfected this plasmid would yield two types of RNA (Fig. PB1-vRNA, synthesized by cellular pol I; and an with a 5′-cap structure, synthesized by the pol II. lation of the mRNA should result in the synthe PB1-protein.

To examine whether the PB1-protein is produced this construct, we tested replication and transcrip an artificial vRNA by constructing the expression mids pHW21-PB2, pHW23-PA, and pHW25-NP. contain cDNAs encoding PB2, PA, and NP prote A/WSN/33 under the control of the CMV-promot the *in vivo* synthesis of an artificial vRNA, we cons the reporter plasmid pHW72-EGFP (Fig. 2A), cont the EGFP cDNA flanked by the noncoding region M-segment and the human pol I-promoter and t rine terminator sequence. Five plasmids (2 μg p PB2, 2 μg pHW52-PB1 [pol I/pol II-promoter const μg pHW23-PA, 2 μg pHW25-NP, and 1 μg pHW72-E were transfected into 293T cells. Twenty-four an after transfection, the cells were analyzed by f cence microscopy. After 24 h, fluorescent cells observed (data not shown). This result shows that 24 h the polymerase proteins are synthesized in centration sufficient to allow recognition of the int virus-specific ends of the EGFP-vRNA. These c synthesize mRNA, which is translated into EGFP.

To evaluate the efficiency of this system, we perf flow cytometric analysis to count the number of fl cent cells (Fig. 2B). Forty-eight hours after transfecti the five plasmids, 18.72% of the cell population sho fluorescence. Only a background level of fluores cells (0.06%) was observed when pHW52-PB1 pla was not added; this finding is consistent with thos earlier studies, which showed that all four RNP-co proteins are necessary for the amplification of the v (Huang *et al.*, 1990). The results indicate that the cDNA transcription and the resulting concentratio PB1 protein together with the other RNP complex teins is sufficient to initiate a viral transcription/rep tion process.

Generation of recombinant influenza A virus

For the generation of infectious influenza A virus necessary that the plasmid pHW52-PB1 provides only PB1 mRNA and protein but also sufficient amo of PB1-vRNA, which can be packaged into progeny v (Fig. 1B). For the remaining seven vRNAs, we used pl mids that contain the cDNAs for the full-length RNAs the A/WSN/33 virus, flanked by the human pol I-promo and the murine terminator. Transfection of these pl

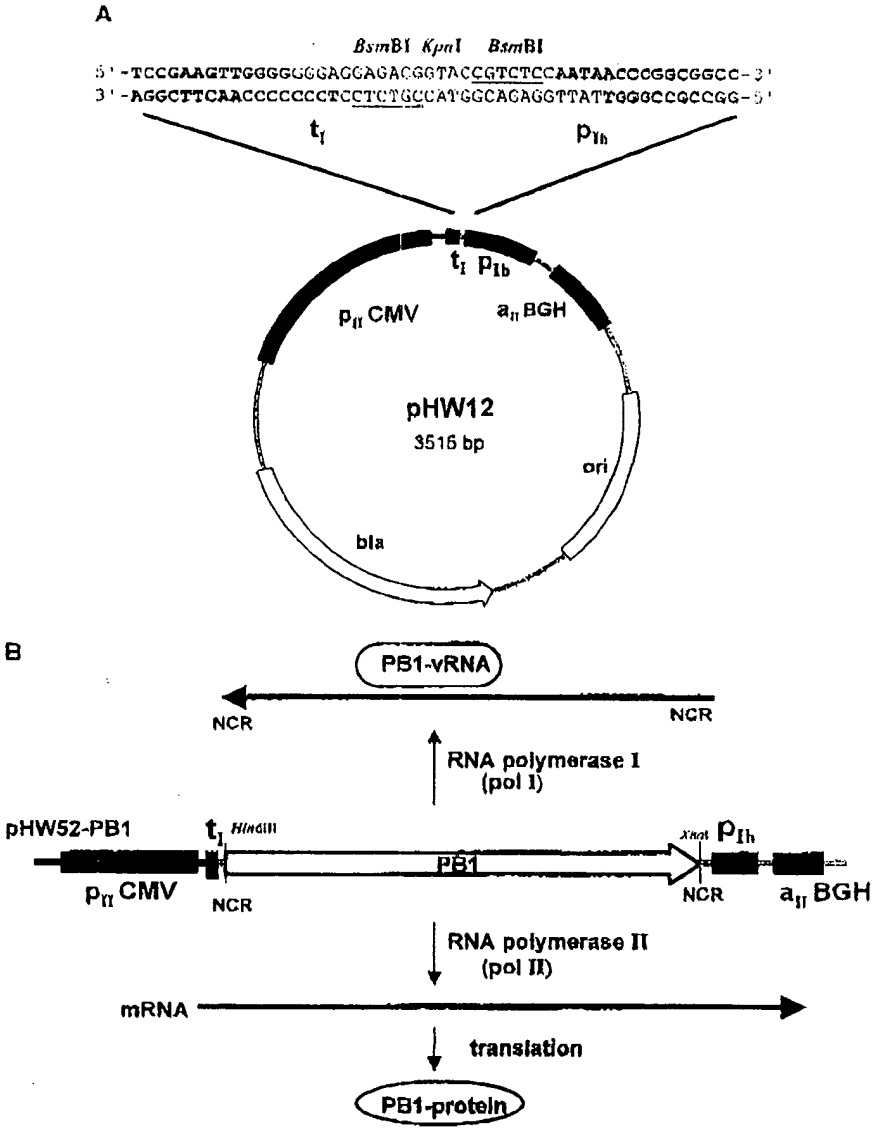

FIG. 1. The pol I/pol II bidirectional transcription system. (A) The cloning vector pHW12. The plasmid contains the RNA polymerase II promoter (p_{II}CMV) of the human cytomegalovirus and the polyadenylation signal (a_{II}BGH) of the gene encoding bovine growth hormone. Inserted between these elements is a murine terminator (t_I) and the sequence of the human RNA polymerase I promoter (p_{Ih}). For insertion of arbitrary sequences between the pol I promoter and terminator, two *Bsm*BI restriction sites were introduced. After digestion with *Bsm*BI, a vector fragment (bold type) with sticky but noncomplementary 5′ protruding ends is generated. For propagation in *E. coli*, the plasmid contains an origin of replication (ori) and a beta lactamase gene (bla) for selection in ampicillin-containing medium. (B) The expression plasmid pHW52-PB1 and proposed transcription products after transfection. The plasmid is derived from pHW12. cDNA of the PB1-segment is inserted between the pol I promoter (p_{Ih}) and terminator (t_I). The arrow represents the sequence for the ORF of the PB1 gene. Between the influenza virus-specific noncoding region (NCR) and the coding region for the PB1 gene, sequences containing *Hin*dIII and *Xho*I restriction sites have been inserted. After transfection of this expression plasmid, two types of molecules are expected to be synthesized. From the human pol I promoter, RNA with the 5′ and 3′ noncoding region is transcribed by cellular RNA polymerase I, and this transcription results in an influenza-like vRNA molecule with recognition elements for the viral polymerase proteins at both ends. Transcription by RNA polymerase II is expected to result in an mRNA with a 5′ cap structure and a 3′ poly A tail; this mRNA expresses PB1 protein after translation.

molds should result in the synthesis of all eight viral RNAs that are replicated and transcribed by the polymerase proteins, forming new vRNPs. After synthesis of the structural proteins, the RNPs would be packaged into new virus particles.

We transfected 293T cells (Table 1) with different

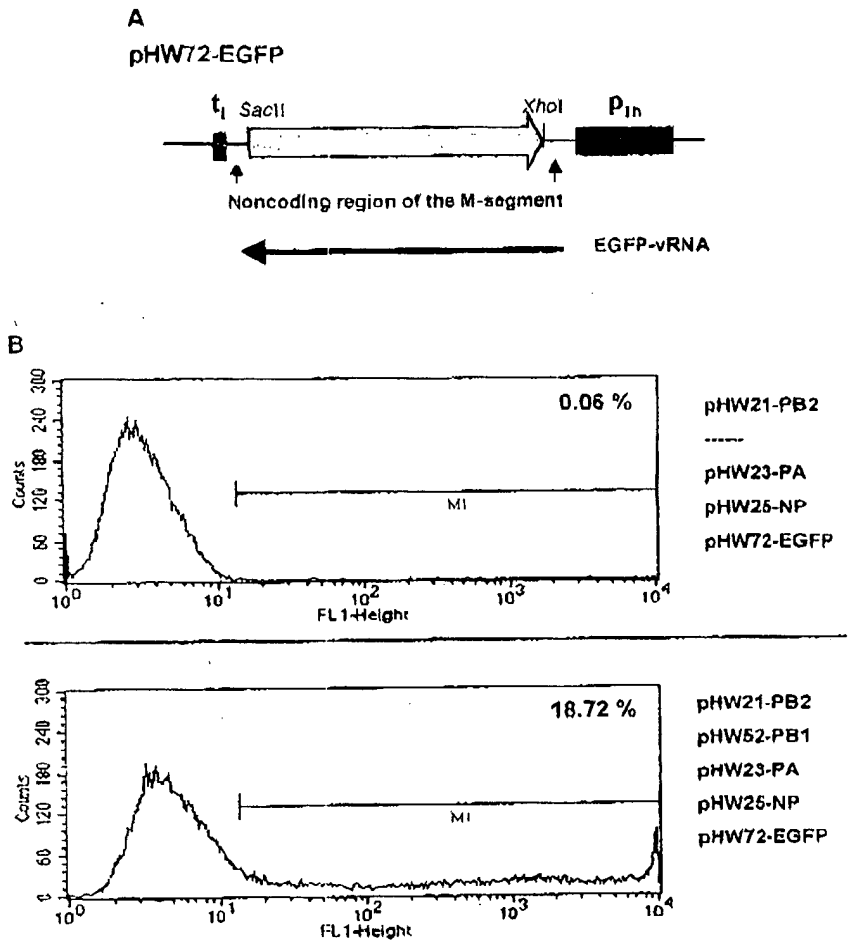

FIG. 2. Flow cytometric evaluation of the efficiency of the plasmid-based pol I/pol II transcription system. (A) Schematic representation of the reporter plasmid pHW72-EGFP with a pol I-transcription unit containing the sequences of the noncoding region of the M segment and the cDNA encoding EGFP. (B) We transfected 293T cells with the indicated plasmids; 48 h later, we used flow cytometric analysis to determine the number of fluorescent cells. Upper panel: flow cytometric analysis after transfection of pHW21-PB2, pHW23-PA, pHW25-NP, and pHW72-EGFP without pHW52-PB1; lower panel: analysis after transfection with pHW52-PB1. The bar M1 represents the region used to determine the percentage of fluorescent cells.

amounts of pHW52-PB1 plasmid (0, 2, and 4 μg) together with the plasmids pPolI-WSN-PB2, pPolI-WSN-PA, pPolI-WSN-HA, pPolI-WSN-NP, pPolI-WSN-NA, pHW127-M, and pHW128-NS (1 μg each). The protein-expression plasmids pHW21-PB2 (1 μg), pHW23-PA (0.1 μg), pHW25-NP (1 μg), pEWSN-HA (1 μg), and pCAGGS-WNA15 (1 μg) were cotransfected. The expression plasmids for the hemagglutinin (HA) and the neuraminidase (NA) were included to increase the yield of transfectant virus.

Forty-eight hours after transfection, the supernatant of the primary transfected 293T cells was transferred to MDCK cells. In all transfection experiments in which pHW52-PB1 plasmid was added, 24 h after the passage we observed a virus-induced cytopathic effect. No cytopathic effect was visible if no PB1-expressing plasmid was included in the transfection reaction. The virus titer was determined by titrating the supernatant of the transfected cells on MDCK cells; the supernatant was found to contain 2×10^4–2×10^5 pfu/ml. This finding shows that, after transfection of the PB1-pol I/pol II-promoter plasmid (together with the expression plasmids), PB1 vRNA and PB1 protein are synthesized in the human cell line 293T at a level sufficient for the generation of infectious influenza A viruses. In the cotransfection experiments (Table 1) with plasmids containing the PB1-cDNA separated on two plasmids (pHW82-PB1 and pHW22-PB1), a virus titer of 2×10^4 pfu/ml was found; the analogous experiment using the plasmids with wild-type PB1 sequences (pPol I-WSN-PB1 and pHW22-PB1) resulted in a virus titer of 3×10^8 pfu/ml.

Unlike the expression construct with a pol II-promoter used in a previous study (Neumann *et al.*, 1999), we used

TABLE 1

Plasmid Sets Used for Recovery of Recombinant Influenza A Virus

Plasmid	Promoter	cDNA	μg-Transfected plasmid DNA				
pHW21-PB2	pol II	PB2	1	1	1	1	1
pHW22-PB1	pol II	PB1	—	—	—	1	1
pHW23-PA	pol II	PA	0.1	0.1	0.1	0.1	0.1
pHW25-NP	pol II	NP	1	1	1	1	1
pEWSNHA	pol II	HA	1	1	1	1	1
pCAGGS-WNA15	pol II	NA	1	1	1	1	1
pPolI-WSN-PB2	pol I	PB2	1	1	1	1	1
pHW82-PB1	pol I	PB1*	—	—	—	1	—
pPolI-WSN-PB1	pol I	PB1	—	—	—	—	1
pPolI-WSN-PA	pol I	PA	1	1	1	1	1
pPolI-WSN-NP	pol I	NP	1	1	1	1	1
pPolI-WSN-HA	pol I	HA	1	1	1	1	1
pPolI-WSN-NA	pol I	NA	1	1	1	1	1
pHW127-M	pol I	M	1	1	1	1	1
pHW128-NS	pol I	NS	1	1	1	1	1
pHW52-PB1	pol I + pol II	PB1*	0	2	4	—	—
Virus titer (pfu/ml)			0	2×10^4	2×10^5	2×10^4	3×10^0

Note. 293T cells were transfected with the indicated plasmids; the virus titer in the cell culture supernatants was determined in MDCK cells.
* The cDNA contains additional noninfluenza A virus sequences in the noncoding region of the PB1 segment.

the plasmid pHW52-PB1 (Fig. 1B) that contains sequences derived from the pol I-transcription unit that are inserted between the CMV-promoter and the polyadenylation site. The expression of the EGFP reporter gene demonstrates that the overall expression of PB1-protein in this system is sufficient for formation of EGFP–RNP complexes. Although the pol I-promoter/terminator region contains recognition sequences for pol I-specific transcription and termination factors (Bell *et al.*, 1988; Kuhn *et al.*, 1994; Beckmann *et al.*, 1995), these DNA-binding proteins do not seem to inhibit pol II-mediated transcription. These findings are consistent with the finding that the pol I-specific DNA-binding proteins are more abundant in the nucleolus, the compartment in which the cellular rDNA-transcription takes place (Evers *et al.*, 1995). These results indicate that after transfection of the pol I/pol II-promoter construct into the cell, some of the plasmids are delivered to the nucleolus, where the pol I-mediated transcription occurs, and some are retained in the nucleus, where they are transcribed by RNA polymerase II.

Because the reporter construct pHW52-PB1 contained additional noninfluenza virus sequences (restriction sites) in the noncoding region before the start codon and after the stop codon, we were interested as to whether these sequences were stably maintained in the viral PB1 RNA segment (Fig. 3A). Therefore, we isolated vRNA after the second passage of transfectant virus on MDCK cells and performed reverse-transcription PCR analysis. As shown in Fig. 3B, the amplification of vRNA with PB1-specific primers resulted in the generation of cDNA fragments of the expected sizes. With the same viral RNA and primers, but without the addition of reverse-transcriptase, no amplification product was obtained, showing that the cDNA originated from viral RNA and not from plasmid DNA carried over from the supernatant of transfected cells. Sequencing of the PCR products revealed that both restriction site sequences were present in the RNA molecule. The results show that the pol I/pol II-transcription system allows recovery of infectious recombinant virus and that virus with foreign sequences in the noncoding region of the PB1 gene is viable. This modified-PB1 segment is still replicated, transcribed, and packaged into virus particles. Previously, by using the RNP-transfection system, the noncoding regions of influenza A virus segments were changed. By substituting the noncoding region of the NA gene with the corresponding sequence of the NS segment of influenza B, transfectant influenza viruses were obtained (Muster *et al.*, 1991; Bergmann and Muster, 1995). This type of virus with a chimeric NA segment showed an attenuated phenotype in mice and protected mice inoculated with a nonlethal dose against infection of the wild-type influenza virus infection. These results showed that the genetic alteration of the noncoding region of an RNA segment can change the biological property of a transfectant virus. Here, we report for the first time that even noninfluenza virus sequences can be inserted into the noncoding region of the PB1 segment. With the pol I/pol II-transcription system, it is now possible to systematically modify these sequence elements in the noncoding region of the PB1 segment and to evaluate whether these genetic

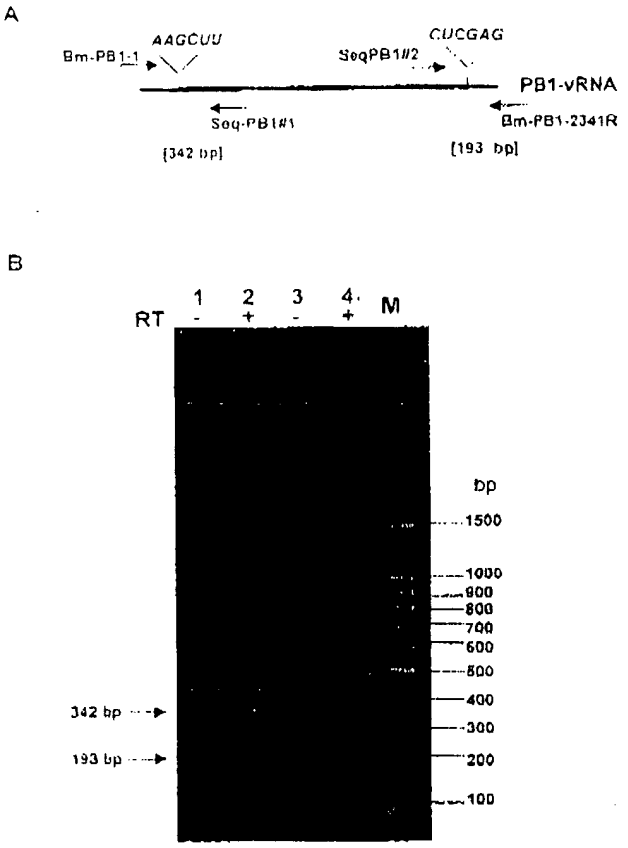

FIG. 3. Detection of recombinant influenza A virus by RT-PCR. (A) Schematic representation of the recombinant PB1 RNA segment and the location of primers used for RT-PCR. The sequences (shown in cRNA orientation) above the RNA segment represent the introduced non-influenza virus sequences before the start codon (AAGCUU) and after the stop codon (CUCGAG). The primers used in the RT-PCR reaction are represented by arrows. The expected sizes of the fragments at the 5′ and 3′ parts are shown in brackets. (B) RT-PCR reaction was performed with primers specific for the PB1 gene segment and with vRNA extracted from virions. The reaction products were subjected to electrophoresis on a 2% agarose gel. To ensure that the amplified DNA fragments were derived from vRNA, and not from plasmid DNA carried over from transfected cells, one reaction was performed without the addition of reverse-transcriptase (−). Lanes 1 and 2: reactions using the primers Bm-PB1-1 and Seq-PB1#1; lanes 3 and 4: reactions using Seq-PB1#2 and Bm-PB1-2341R; M: 100-bp ladder (Promega). The presence of the inserted sequences was verified by sequencing the amplified DNA fragments.

manipulations result in changes in the biological properties of the recombinant viruses. Indeed, the lower yield of the viruses with the mutated PB1 segment compared to the wild-type virus indicates that the inserted sequences negatively influence the virus growth.

Although the plasmid-based system developed recently (Neumann *et al.*, 1999) is highly efficient in generating influenza virus, it involves cloning of 14 to 17 plasmids. In this study, we reduced the number of plasmids to 13, which are needed for the efficient recovery of influenza A/WSN/33 virus strain. The reduction in the number of plasmids achieved by this approach promises to increase the efficiency of transfection for cell lines other than 293T cells, thus allowing the delivery of genes to cell lines for which the efficient delivery of 14 plasmids is difficult to achieve. Fodor *et al.* (1999) were able to rescue influenza virus after transfecting 12 plasmids; the virus yield in this study was 1–2 infectious virus particles per 10^6 transfected Vero cells. It would be interesting to investigate whether the pol I/pol II-transcription system, in addition to the transcription of PB1-cDNA, can also be used for the remaining seven RNA segments. Such investigations could test whether the pol I/pol II bidirectional transcription system allows the development of a plasmid-based system that contains eight plasmids for the production of influenza A viruses, thus improving this reverse-genetics system by decreasing the time and cost required for genetic engineering of influenza A viruses. It would also be interesting to investigate whether the transfection system containing a pol I- and pol II-promoter on the same plasmid is applicable for the generation of other orthomyxoviruses (i.e., influenza B, thogotovirus).

MATERIALS AND METHODS

Cloning of plasmids

All cloning and PCR reactions were performed according to standard protocols. Briefly, the expression plasmids for the polymerase-complex genes of A/WSN/33 were derived from pcDNA3 (Invitrogen, La Jolla, CA) containing the immediate early promoter of the human cytomegalovirus (CMV) and the poly A site of the gene encoding bovine growth hormone (BGH). The viral cDNAs were derived from the plasmids pWNP143, pWSNPA3, pWSNPB2–14, and pGW-PB1 (kindly provided by D. Nayak) to yield the expression constructs pHW25-NP, pHW23-PA, pHW21-PB2, and pHW22-PB1, respectively. pHW12 was generated by inserting human pol I-promoter and terminator sequences between the pol II-promoter and the polyA-site (Fig. 1A). The plasmid pHW52 was derived from pHW12 by first inserting oligonucleotides containing the noncoding region of PB1 extended by *Hin*dIII and *Xho*I sites and then inserting the PB1-coding region from pHW22-PB1 into these sites (Fig. 1B). The plasmid pHW82-PB1 was derived from pHW52-PB1 by deletion of the CMV-promoter sequences. The coding region for the enhanced green fluorescent protein (EGFP) in the reporter construct pHW72-EGFP was obtained after PCR-amplification using pEGFP-N1 (Clon-Tech, Palo Alto, CA) as template and inserting the cDNA after *Sac*II/*Xho*I digestion into the plasmid pHW72 (E. Hoffmann, unpublished) containing the human pol I-promoter and murine terminator and the noncoding region

of the M-segment separated by *Sac*II/*Xho*I sites (Fig. 2A). pHW127-M and pHW128-NS were constructed by RT-PCR amplification of viral RNA with the primers containing segment-specific sequences and *Bsm*BI sites for insertion into the *Bsm*BI-digested vector pHH21 (Hoffmann, 1997; Neumann *et al.*, 1999). All sequences of the oligonucleotides for PCR amplification or cloning are available on request. The construction of the plasmids pPolI-WSN-PB1, pPolI-WSN-PB2, pPolI-WSN-PA, pPolI-WSN-NP, pPolI-WSN-HA, pPolI-WSN-NA, pEWSN-HA, and pCAGGS-WNA15 has been described elsewhere (Neumann *et al.*, 1999).

Cell culture and transfection

Madin–Darby canine kidney (MDCK) cells were maintained in modified Eagle medium (MEM) containing 10% fetal bovine serum (FBS); 293T human embryonic kidney cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS. TransIT LT-1 (Panvera, Madison, WI) was used according to the manufacturer's instructions to transfect 1×10^6 293T cells. Different amounts of plasmids (Table 1) were mixed with TransIT LT-1 (2 μl TransIT LT-1 per 1 μg of DNA), incubated at room temperature for 45 min and added to the cells. After 6 h, the DNA-transfection mixture was replaced by Opti-MEM (Gibco/BRL, Gaithersburg, MD), containing 0.3% bovine serum albumin (BSA) and 0.01% FBS. Forty-eight hours after transfection, supernatants containing virus were titrated in MDCK cells.

RNA isolation and RT-PCR

Viral RNA was isolated from virus particles with the use of the RNeasy-Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For characterization of recombinant influenza viruses, the Access RT-PCR kit (Promega, Madison, WI) was used according to the protocol provided. The following primers were used in the RT-PCR experiments: Seq-PB1#1: 5′-AGG ATG GGA TTC CTC AAG G-3′; Seq-PB1#2: 5′-GCT ATG GTT TCC AGA GCC CG-3′; Bm-PB1-1: 5′-TAT TCG TCT CAG GGA GCG AAA GCA GGC A-3′; Bm-PB1-2341R: 5′-ATA TCG TCT CGT ATT AGT AGA AAC AAG GCA TTT-3′. RT-PCR experiments were performed by using the PTC-200 DNA engine (MJ Research, Watertown, MA). The amplification program started with one cycle at 48°C for 45 min (first-strand cDNA synthesis) and one cycle at 94°C for 2 min (inactivation of the AMV reverse-transcriptase and cDNA denaturation). These cycles were followed by 40 cycles at 94°C for 20 s, 52°C for 30 s, and 72°C for 30 s (PCR amplification); the program ended with one cycle at 72°C for 5 min. The PCR products were analyzed by agarose gel electrophoresis and sequenced with the primer Seq-PB1#1 or Seq-PB1#2 (Fig. 3).

Flow cytometry

Forty-eight hours after transfection, 293T cells were washed with phosphate-buffered saline (PBS), pelleted, and resuspended in PBS plus 5% FBS. Flow cytometric analysis was performed by using a FACSCalibur flow cytometer (Becton-Dickinson) and the data were analyzed by using the CellQuest software package. For EGFP expression analysis, we used the emission wavelength of 530 nm (FL1) to achieve a high sensivity for EGFP-mediated fluorescence detection.

ACKNOWLEDGMENTS

These studies were supported by Public Health Research Grants AI29680, AI08831, AI29599, and AI44388 from the National Institute of Allergy and Infectious Diseases; by Cancer Center Support CORE Grant CA-21765; and by the American Lebanese Syrian Associated Charities. We thank S. Krauss, D. Walker, and D. Todd for excellent technical support and Dr. R. Cross for performing the flow cytometry. We also thank Julia Cay Jones and Flo Witte for scientific editing.

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